

Double-talk after Reykjavik

Both the Soviet Union and the United States have evidently over-committed themselves to arms control proposals that cannot be safely carried through. They need help in backing away from them.

THE fallout from Reykjavik has been confusing and disturbing. Two weeks ago, the general sense of failure appeared to be shared both by the Soviet Union and the United States. Then, the word went out, the hurried meeting at Reykjavik had not been a failure at all, but a successful exploration of radical measures of arms control whose negotiation would be completed at the bilateral negotiations under way at Geneva. This claim, on the acceptance of which the reputation of each government depends, is unfortunately disingenuous. The meeting was advertised as one at which arrangements would be made for a less hurried meeting later in the year, but no arrangements were, in the event, made. And while it is plain that some of the arms control proposals discussed at Reykjavik were radical by previous standards, it is also clear that, in their different ways, they are almost insurmountable obstacles to agreement.

The Soviet Union's eye-catching plan, of which Mr Mikhail Gorbachev has been singing since the beginning of the year, that all strategic nuclear weapons should be "eliminated" within a decade is, strange as it may seem, one obvious stumbling block. Seductive though the idea may be that the clock should be turned back to before 1945, when nuclear weapons were first used strategically to bring the Second World War to an end, that gigantic assault on the historical process cannot be undertaken in the light-hearted manner that Mr Gorbachev suggests. There may be a case for doing away with strategic nuclear weapons, but that would not by itself ensure that we would all sleep easier in our beds at night. For that to be possible, the following questions cannot be dodged:

● **What assurance would there be that neither of the superpowers would keep nuclear weapons hidden against an emergency?** Only the comprehensive inspection of all military nuclear installations could provide the security for which both sides would look, but could the negotiation of such arrangements be completed in a mere decade?

● **What would be done, by superpowers innocent of strategic nuclear weapons, about threats by smaller nuclear powers?** There are already three smaller powers in this category (Britain, China and France), not to mention others waiting in the wings. The logical solution would be to negotiate the general abandonment of strategic nuclear weapons. It is anybody's guess how long that would take, given that, so far, nobody has tried.

● **How, in the absence of strategic nuclear weapons, would conventional conflicts in, say, Europe be settled?** Presumably, under Mr Gorbachev's proposal, the present superpowers would be free to intervene in (or to attempt to deter) such conflicts by the use of tactical nuclear weapons, which is the state of affairs with which Europe was uneasily familiar in the early 1950s. The obvious difficulty is that the present threat of mutually assured destruction (the bizarre basis of the theory of deterrence) would be replaced only by the assurance that the superpowers might engage in nuclear conflicts without suffering much themselves. The only stable state of affairs would entail the abolition of tactical nuclear weapons as well, a great but distant dream.

These are only some of the reasons why Mr Gorbachev's proposal is unworkable, at least within the timescale he has laid

down. Much the same is true of the counter-proposal from the United States produced at Reykjavik like a rabbit from a hat. The proposal is that all ballistic missiles should be abolished, and that the superpowers should rely on more primitive (and vulnerable) ways of delivering nuclear weapons if the need should arise. This, again, is tantamount to a return to the early 1950s. The threat of nuclear retaliation against a transgressing power would still be credible, but retaliation would be no swifter than the passage time of aircraft and cruise missiles and no more sure than the deficiencies of the other fellow's air defences. Nuclear deterrence would become a kind of lottery.

Nobody should be surprised that European governments have been so quick to tell Washington that they are as uneasy about this proposal as about Mr Gorbachev's scheme. Under each scheme, they would have to put up with the knowledge that the United States would not be as directly committed to Europe as it is at present, even though the difference would not be very great; put crudely, soldiers are more evocative hostages than missiles. Yet the US proposal would be acceptable to the Soviet Union only if at least the British and French missiles were negotiated away, which is doubly unlikely in the circumstances.

These proposals, outwardly well-intentioned, have now become obstacles to further progress simply because they have been made. Neither side will wish to withdraw to a more moderate position for fear of being accused by the other of having been insincere at Reykjavik. Yet the world would be better off with, say, an interim agreement to reduce strategic warheads by a half and to abolish missiles of intermediate range in Europe, the sort of compromise talked of at Geneva in the past few months. Why could not the United States and the Soviet Union have agreed to sign such a package two weeks ago — and to keep on talking?

The other conspicuous obstacle is the Strategic Defense Initiative (SDI), on which President Reagan's vision is fixed and fixated. This project is now in a curious limbo of its own. Whether or not it is technically realistic, SDI is by common consent a long-term project that could not come to full fruition for many decades. Even the short-term by-products (such as satellite-based early warning systems) are not just around the corner and are, in any case, relatively uncontroversial. The snag is that President Reagan cannot be seen to back away from his commitment to the concept of an effective defence against ballistic missiles, while the Reykjavik meeting has only sharpened the long-standing argument about the interpretation of the Anti-Ballistic Missile Treaty. (Does a hole in the ground beneath the Nevada desert count as a "laboratory" in which research may be conducted?) Here again, the need is that people should back down a little. It may be easier for Mr Reagan after next month's elections, when he and other supporters of SDI will have an interest in unpackaging SDI to make it more acceptable (and thus less vulnerable) to Congress, perhaps by specifying a series of stages in which successive goals will be attained. At that point, the Soviet Union may also find the project more palatable. Meanwhile Mr Gorbachev and Mr Reagan might usefully reflect what business people are always being told, that it is unwise to attempt important agreements within a few hours of stepping off a long-distance aircraft. □



The other big bang

The City of London is entering the twentieth century awkwardly and apprehensively.

MONDAY this week ranks somewhere between a red-letter day and a nightmare for the City of London, equally well-known as the site of most of Christopher Wren's churches and for the much bigger but less tasteful buildings that now house British and international financial institutions. The occasion was dictated by events two years ago, when the British government reached an accommodation with the London Stock Exchange that offered the largest financial market in Europe immunity from anti-trust investigation for unfair trade practices (fixing commission rates on sales of stock) in return for an undertaking that these practices would be abandoned.

To its credit, the London Stock Exchange has, if anything, over-fulfilled its undertaking. Not only are members of the exchange now allowed to charge what commissions they (and their customers) agree, but the membership of the exchange has been broadened and the old restrictions on who may provide what services in the complicated transaction of the purchase and sale of stock have been abandoned. More than that, financial institutions wishing to do business on the London Stock Exchange are now required to be properly computerized. Those in the business of trading in securities, for example, are required to advertise on a generally accessible computer network the prices at which they are prepared to buy and sell, and then to stand by those prices if a customer should come along. Much in the spirit that there can be nothing about the future than cannot be accommodated within a day's work, these arrangements were all to be introduced in earnest at 0900 GMT on Monday of this week. This, by the legend of the past few months, was the City of London's own big bang.

Inevitably, the worst fears of the sceptics seem to have been instantly confirmed. At the appointed time, the spanking new central computer system meant to make everybody's quotations generally available was out of action, overloaded by a torrent of information from dealers marking their cards for the first time. That and an older supplementary computer system providing information about the prices at which deals have been struck appear to have been in and out of action for most of the first two hours of trading. In retrospect, dealers of legendary brashness will probably agree with what systems analysts have been telling them for the past several months, that even high technology requires proving before use. Their immediate response was traditional, to make the gap between buying and selling prices so huge that trading was discouraged, which is hardly the way to make a fortune especially now that commissions are a smaller proportion of sales.

The more serious defect of this week's arrangements is that the regulations by which the British government plans to control the more competitive financial markets are not yet in place. Part of the trouble is that the British House of Commons has been away since the end of July, with the result that the Financial Services Bill is unlikely to become an act for some weeks yet. More seriously, the basis on which the planned regulations have been drawn is an insecure compromise between formal legislation and the City's traditional informality. The intention is that the probity of the City's financial institutions will be assured by a system of 'self-regulation' under which groups of similar institutions will agree to promulgate rules by which they must all behave. Such a system worked well enough when the functions of financial institutions and people were tightly circumscribed in such a way that conflicts of interest were fewer than they will be now. One sign that the government acknowledges the difficulty is its creation of a body to supervise self-regulation, a kind of contradiction in terms. This is the body that has decreed that when a financial institution may be privy to private information about one company's plans to buy another, a "Chinese wall"

should separate those concerned from those within the same organization who might make money from the information.

The prospect that such an arrangement will function as intended, as an assurance to the generality of those who deal with London institutions that the insiders do not have an inside track, must be very slim. One big scandal will be enough to blow down the cumbersome system now being installed. The underlying weakness of the planned arrangements is that they are at once unstable and in conflict with the whole objective of the deregulation of the London markets, which is to attract more business from elsewhere in a booming world. The City is fond of the slogan *Caveat emptor* but may yet find that buyers will exercise the caution demanded of them by taking their business elsewhere so long as the protection of investors seems to rest formally with those who make money from their custom. That would be a pity after all this week's excitement. □

Boycott of SDI

Few are fond of the Strategic Defense Initiative, but a boycott of research would be mistaken.

DECLARATIONS of intent never to work for the US Strategic Defense Initiative (SDI) Organization have spread from the United States to Britain (see p.747), and will not stop there. Broadly speaking, they will do no harm and probably a little good, if only by reminding the world at large that many well-informed and well-intentioned people believe that SDI is a waste of their time and other people's money. But there are awkward questions that should be faced by those who will be asked, in the next few weeks, to add their names to the well-filled dotted lines. Here are some.

From the start of the boycott campaign, it has been supposed that academics will be in the front line, and that those working for industrial research organizations will mostly be unable to commit themselves. That is as it should be, but implies two painful truths. First, the campaign against SDI can be directed on moral grounds against the character of what is planned, for then it would be proper that even industrial researchers should be persuaded to join the boycott. That would be justifiable if, for example, the military were looking for people to work on the manufacture of biological weapons in defiance of an international treaty. The organizers admit that SDI is not in that case by saying that their quarrel is not so much with the dangers it presents (chiefly in the context of bad feeling between the Soviet and US governments) as with the belief that it will not work as planned. But that raises the dilemma of whether it is wise to turn away research money, perhaps when there is none else available, even though there is no prospect of a successful outcome and every prospect that the research itself will be technically interesting.

The moral should be plain. Academic researchers, the recruiters' natural targets, should now as always decide for themselves what research they will undertake. The best topics are the most interesting topics, those most likely to yield understanding of the natural world. SDI topics may often qualify. But choosing for or against SDI research on other grounds (money or politics, for example) is improper (and universities should corporately protest at signs that academic researchers follow such lines of argument). If that principle is kept, the assertion that boycotting SDI is somehow disloyal (in the West) melts away; academic patriotism is the pursuit of the greatest excellence. For people who work in unrelated fields, to whom the dilemma will not be immediate, joining the boycott is an empty gesture but a way of making an interesting point, as if the Pope were to sign a declaration by those not intending to join some society of atheists. The danger in the boycott is merely that it might become coercive, relegating to the doghouse people who, for good reason, of their own, prefer not to take part in such a demonstration. □

Strategic Defense Initiative

British echo US boycott of star wars research

MORE than 40 British universities have applied to participate in the research programmes of the US Strategic Defense Initiative (SDI) and six are involved in the nine major contracts so far awarded in the United Kingdom, benefiting from a share of a budget that now totals £25.9 million.

The figures are those of the Ministry of Defence's SDI Participation Office in London as a response to a proposed boycott of the project by half of Britain's academic physicists, who publicly voiced their disapproval of the programme last weekend. The physicists were highly critical of the value of SDI research, though not objecting to defence-related programmes in general.

The British SDI boycott pledge, signed by 545 researchers including three Nobel laureates in physics and 25 Fellows of the Royal Society, is meant to endorse the stand taken by academic researchers in the United States. More than 7,000 academics have signed the US pledge, among them 3,700 science and engineering professors and senior researchers, as well as 57 per cent of physics faculties at the top 20 research universities.

Neither the US nor the British signatories will accept or solicit funds for SDI research. According to the British partici-

pants in the weekend's meeting, their support is necessary to prevent their US colleagues from being isolated and pressurized into unacceptable research programmes. They say the pledge is initially meant to "document and publicize" the scale of opposition to the SDI programme by academic researchers, science and engineering lecturers and graduate students.

They further claim that the boycott will impede the development of what they believe to be a dangerous programme and will alert the scientific community to the role it has been asked to play in this "fraudulent peace shield".

It will also serve, they say, as a means of fostering discussion among researchers about SDI and the political profile that has been forced on the work of those already engaged on the programme. The British organizers have called on those now involved in research to think again and withdraw from the programme.

The British pledge reads: "We, the undersigned, believe that the Star Wars programme is technically dubious. An anti-ballistic missile defence system of sufficient reliability to defend populations against a Soviet first strike is not technically feasible in the foreseeable future . . . According, as working scientists, we will not apply for or accept support from the Strategic Defense Initiative Organization, which funds Star Wars research. We encourage other scientists and technical personnel to join us in refusing to cooperate with this deeply misguided and enormously expensive programme." **Bill Johnstone**
• Tim Beardsley adds from Washington: The SDI boycott originated in the United States, principally on the campuses of Cornell University and the University of Illinois at Urbana-Champaign. The wording is essentially the same as the British version, committing signers of the pledge neither to solicit nor to accept funds for research related to SDI.

Separately, an open letter to Congress expressing "great reservations" about the wisdom of pursuing SDI has been signed by more than 2,000 researchers in industry and at national laboratories.

It is hard to estimate how far SDI research has actually been affected by the pledge, but John Kogut, a physics professor at the University of Illinois and one of the instigators of the campaign, points out that at Cornell University, 52 per cent of signatories had previously accepted support from the Department of Defense and so were potential SDI researchers.

The Department of Defense has said it has no shortage of good research propo-

Soviet Union

Planning the riposte to SDI

THE Soviet Union would take "adequate but not identical" countermeasures if the United States decided to deploy its Strategic Defense Initiative (SDI), Mr Gennadii Gerasimov, head of the information directorate of the Soviet Ministry of Foreign Affairs, announced last week. He suggested that a financial analysis for such measures had already been carried out. The cost, Gerasimov said, works out significantly less than that of the SDI programme and quite within Soviet capabilities. Some scientists, he stated, put it as low as 5 per cent of the calculated US expenditure on SDI.

Soviet spokesmen, both before and after the Reykjavik talks, have been careful to stress that the Soviet Union has no SDI-type programme of its own. Since Reykjavik, however, there have been several indications in the Soviet media that the Soviet Union is prepared to undertake appropriate anti-SDI measures. Interviewed on Soviet television, Academician Konstantin Frolov, vice president of the Soviet Academy of Science, warned that "if the United States goes beyond the conditions of laboratory research on new types of weapons under the SDI programme, then of course the scientists of the Soviet Union will find the same approaches, and, of course these chances will always even out".

The relationship between Frolov's "similar approaches" and Gerasimov's "adequate but not identical" approach is not clear; Gerasimov is particularly insistent that the Soviet Union has no plans to work on a nuclear-pumped laser. He told *Pravda* last week that it would in any case be impossible to carry out such research as the Soviet Union has declared a moratorium on nuclear tests.

This moratorium is, of course, one of Mr Gorbachev's most valuable cards in the negotiating process, and not one he is likely to abandon lightly. Whatever form counter-SDI measures may take, it seems unlikely, to judge from Gerasimov, to be laser-based.

Vera Rich

Missing — not dead

Dr Mohammed Younis Akbari, an Afghan nuclear physicist, who was sentenced to death by a revolutionary court in May 1984, is apparently still alive. A message from a fellow-prisoner relayed to Europe last month claims that Dr Akbari is still in prison, although no details are available about the conditions under which he is being held.

Akbari was sentenced on charges of participating in an illegal political organization and of receiving money from the People's Republic of China to buy arms. He was, in fact, a member of the Rehaili ('freedom' group) led by a Dr Faiz. According to Akbari, it was Faiz who was offered, and may have accepted, money from the Chinese. He said he had travelled to China, but only for medical treatment.

At the time of his arrest and conviction, Western human rights campaigners pleaded for clemency on his behalf. The latest news, sparse though it is, and the improved situation in Afghanistan (as evidenced by the withdrawal of 8,000 Soviet troops) inspired the establishment, last month, of a new "Akbari Clemency Group".

Vera Rich

sals. But the pledge has demonstrated the extent of opposition on campuses in the United States, and the SDI organization has grown noticeably more reticent about research contracts with academics.

Kogut, who has lectured on SDI several times in Britain, says the idea for a British version of the pledge arose out of discussions between him and Ann Davis of the University of Cambridge, among others. Kogut has hopes of taking the pledge to other countries also, and says support is particularly strong in West Germany. □

In vitro fertilization

Research in Victoria threatened

Melbourne

THE state government here in Victoria has virtually brought to a halt research on *in vitro* fertilization (IVF). The Infertility Medical Procedures Act, which came into force in August, carries penalties of up to four years' imprisonment for those carrying out certain types of research; procedures that entail the destruction of, or damage to, a living embryo are forbidden.

Dr Alan Trounson, head of the research group at the Centre for Early Human Development at the Queen Victoria Hospital here, says that his 12-member team will go overseas within six months unless it can be exempted from the provisions of the law. This will be especially ironical because the collaboration between Trounson and Professor Carl Wood, head of obstetrics and gynaecology at the hospital, is widely believed to have given Australia an outstanding position in IVF techniques. The team was the second in the world to deliver a 'test-tube baby' and the first to en-

gineer a successful pregnancy from a frozen embryo.

The essence of the new act is that a human egg once fertilized must be allowed to develop to maturity. Trounson says the law gives a fertilized egg the same status as a newborn baby. Cloning of embryos is also forbidden. Embryos may be created only at the request of couples enrolled in an IVF programme, in which case embryos must be implanted in the woman and reared to maturity.

The law may allow some observations on spare embryos, but these are likely to be embryos which have been frozen and which the prospective parents have decided not to use. Proposals for such investigations will have to be approved by the Standing Review and Advisory Committee on Infertility chaired by Professor Louis Waller, who was also chairman of the committee that recommended the new law to the government of Victoria (see *Nature* 311, 289, 1984).

Trounson says that the law, as it stands, entirely prevents the assessment of experiments researchers wish to carry out. He cites as an example a proposal to investigate the effects of freezing on human eggs, which are more liable than embryos to be damaged, which is now being considered by the Waller committee. Trounson says that the only way of assessing the viability of unfrozen eggs is to attempt to fertilize them. The researchers say they are in a cleft stick: their own ethics committee will not allow them to use untested techniques clinically, but the new law and the Waller committee insist that an embryo can be created only if intended for clinical use.

Trounson's one ray of hope is that the section of the bill forbidding the use of embryos for research was not included in the enacted version, but is the subject of further representations to be made to the state parliament. Trounson's present frustration is the length of time being taken to discuss what he considers his team's legitimate need for an exemption from the strict provisions of the disputed clause. He says that "if you have an inflexible law and an inflexible committee, there is no chance of a resolution".

This is the spirit in which Trounson has spelled out his six months deadline, which he says is the longest time for which he can hope to keep his team together. One member has already left, and Trounson says that none would have difficulty in finding a job elsewhere. But Trounson would prefer to keep his team intact. He says there have already been firm offers from Britain and Canada.

Another IVF research group has already gone. In August, Dr John Kerin's IVF research team of five from the University of Adelaide in South Australia moved to Los Angeles to join the group headed by Dr Richard Marrs at the University of California, Los Angeles, and based at the Cedars Sinai Medical Center. Kerin's group was not forced out of Australia by the prospect of law changes, but lured away by the offer of attractive working conditions; as yet, there is no legislation on IVF in South Australia.

That may not be the case much longer. Legislation is now being considered by the federal parliament to extend Victoria's restrictions nationwide. A Senate committee report on the Human Embryo Experimentation Bill earlier this month recommended imprisonment for the destruction or cloning of embryos. Although the report supports "therapeutic" research intended to improve the chances of the development of the human embryo, it would ban "non-therapeutic" or destructive experimentation. The terms "therapeutic" and "non-therapeutic" are taken from the Helsinki convention on human experimentation. What researchers fear is that this may be yet another Catch-22.

Charles Morgan

US engineering

Defence demands no problem

Washington

CONCERNED that the current military build-up might be swallowing up available engineering talent, leaving civilian industries high and dry, a panel of the National Academy of Engineering last spring began an investigation of how well the supply of engineers was meeting the demand. In its final report*, released last week, the panel concluded not only that defence needs for engineers are not overwhelming the supply, but that the current increase in defence outlays is not a large one by historical standards.

With the advent of the Strategic Defense Initiative (SDI) in addition to the Reagan administration programme to restore US military strength, conventional wisdom held that young engineering talent would inexorably be drawn to lucrative careers in defence industries. But Harrison Shull of the University of Chicago, who chaired the panel on engineering labour markets, says this has not happened. Except for certain subspecialties companies are not having trouble filling technical positions. Traditionally, between 20 and 30 per cent of engineers work in defence-related industries, a figure that will probably remain stable.

The panel found salaries in defence and civilian industries are equivalent in most areas, suggesting no need to compete for talent. The panel reached no firm conclusions about whether the "best" engineers were evenly spread through industry, but noted that there is today less aversion to

defence projects than there was two decades ago. Although Defense Department projections may point toward a continued increase in defence spending, budget realities suggest that defence

It's a Rail Gun adapted to fire money at engineers...



spending as a percentage of gross national product has levelled off. If that is so, then the greatest impact of the recent defence build-up has passed with no major disruption of the engineering marketplace.

If the current situation is stable, signs for the future are harder to interpret. Neither available databases nor predictive models are adequate to assess potential problems in the supply of engineering talent. The international character of engineering services must also be considered in future evaluations of the problem. Shull notes that some US companies have already begun to look overseas for solutions to engineering problems.

Joseph Palca

* *The Impact of Defense Spending on Nondefense Engineering Labor Markets* (National Academy Press, Washington, DC).

US biomedical research

Hughes money talks volumes

Washington

In 1975, the Howard Hughes Medical Institute spent less than \$3 million on biomedical research. Today the annual expenditure is more than \$200 million, and will exceed \$300 million by the end of the decade. The institute is now a major national player in the field, and one that is bringing about a fundamental change in how US biomedical research is conducted. As might be expected of an institute with a distinctive formula for supporting research, it has not gone uncriticized.

The rapid expansion has gained pace in the past two years, since the institute's board of trustees decided to sell the Hughes Aircraft Company, of which it was the sole owner. The company was sold on 4 June 1985 to General Motors for around \$5,000 million. Since then, the institute has embarked on a massive expansion of its research laboratories, improving facilities at existing laboratories and negotiating to provide space for new ones. The two most recent are at the University of California at Los Angeles and at Rockefeller University in New York, bringing the total to 22.

At present, Hughes laboratories have by law to be associated with a hospital or some equivalent institution. Some researchers have expressed concern that the very size of Hughes' resources could cause an undesirable 'brain drain' from non-clinical to clinical research centres. Academic biologists at non-clinical institutions fear that Hughes policies could lead

to a resurgence of the 1960s dominance of biomedicine by physicians. But according to the institute's president, Dr Donald Fredrickson, former head of the National Institutes of Health (NIH), things may soon change. The institute has been wrangling for much of this year with the Internal Revenue Service on the interpretation of its legal obligation to be engaged in the "direct active conduct of research" in conjunction with a hospital. Fredrickson thinks that that definition, which dates back to the 1950s, may not be applicable to today's conditions.

Another fear sometimes voiced by academics is that Hughes, by generously supporting large laboratories at a few select institutions, could distort research by creating 'black holes' into which many of the best scientists would disappear, never to be seen outside again. So Hughes, anxious to avoid such criticisms, plans to spread its influence wider and thinner than in the past, by establishing 'mini-laboratories' comprising one or a handful of investigators. And, depending on the outcome of the legal arguments, Hughes hopes to become an important supporter of research students. Already, a small programme is in place that sends 35 medical students each year to get research experience at NIH.

Because Hughes money is concentrated in specific research areas — and fashionable ones at that — its influence in them is proportionately greater. Research so far has been principally in genetics, immunol-

ogy, neuroscience and cell biology and regulation; Hughes is also taking the lead in supporting several databases of genetic maps. But it now plans a major expansion of its support for structural biology.

The controversy Hughes money is stirring in the research community is at least in part because of the peculiarly generous terms under which support is offered. Recipients are selected on the basis of track record only; not even a research proposal has to be submitted. But once a researcher becomes a Hughes Investigator (necessary because of legal niceties attached to the institute's status as a Medical Research Organization rather than a foundation), Hughes pays salaries and meets all research costs for up to seven years before renewal. Even postdoctoral researchers can expect an unhindered three years' support. And a Hughes investigator is completely free to pursue his own interests, provided annual reviews approve continuation of support.

Hughes also provides equipment for its laboratories over and above that required for its own employees, so there is spare capacity for use by other researchers at the host institution. Expensive items such as automated DNA and protein sequencers are now being bought by Hughes at quantity discounts. Small wonder that most of those approached have no hesitation about accepting a Hughes offer: NIH cannot approach that sort of support.

Fredrickson says Hughes goes out of its way to avoid disrupting normal academic life. Salaries are the same as would be paid normally by the host university or hospital, and though Hughes investigators must have their own research space, collaboration with others is encouraged.

Researchers who are not fortunate enough to be supported from Hughes' bottomless moneybag may therefore be competing year by year for NIH research grants alongside carefree Hughes researchers doing work of comparable importance and quality, a potentially explosive situation. Conflicts over grant support might in future force NIH to start taking account of who is receiving Hughes money in their own funding decisions, according to Stuart Orkin of Children's Hospital Medical Center, Boston, a new Hughes investigator.

Both Hughes and government research officials stress that there is no deliberate competition between the institute and NIH. But Hughes is now undeniably so important a player that all government agencies are having to watch what it is doing.

The sole criterion for supporting a project, Hughes says, is academic excellence, with an emphasis on the unconventional. Hughes' decisions seem likely to be major influence on the development of biomedicine for the rest of the century and beyond.

Tim Beardsley

Engineered organisms

Keeping track of the wild

Washington

US administration officials are planning a new computer database to provide information for evaluating environmental releases of genetically modified organisms. The information would be available to both researchers and government agencies working on what has become the most vexed regulatory issue in biotechnology.

The idea is to devise a system that would allow rapid access to information on species introductions and microbial interactions already available in the ecological and agricultural literature. In the process it is hoped the exercise would indicate the kind of new research that is most needed.

The plan, which has not yet been approved, has been put forward by a subcommittee on research needs of the Biotechnology Science Coordinating Committee, based in the White House's Office of Science and Technology Policy. The problem at the moment, according to David Kingsbury, chairman of the committee, is that

"we don't know what we know".

The proposal has been discussed with the National Library of Medicine. The library has extensive experience running a database called TOXNET, which stores information on 4,000 hazardous chemicals. According to Dr Dan Masys, director of the library, the data structures and methods of interrogation devised for TOXNET could be adapted easily to environmental release of altered organisms.

The library has ambitions of its own to develop databases relevant to the new biotechnology, possibly in the future linking environmental and molecular biology data in the same system. Such a scheme is still a long way off, but there is growing interest in the idea; the Howard Hughes Medical Institute, among others (see above) is interested in a similar grand linkup of databases. In the meantime, a meeting of information specialists is planned to discuss the environmental database proposal before next April.

Tim Beardsley

European microelectronics

Setting sights on US and Japan

EUROPEAN microelectronic engineers and physicists are making a new assault on the international silicon manufacturing and design industry, now dominated by the United States and other Pacific Basin economies. That seems to be the common thread linking three projects recently unveiled.

One of these developments is the responsibility of a new venture, now but a year old, called European Silicon Structures (ES2), which says that it is now ready to make its first delivery of microchips to customers. The designs, manufactured at Exel in San Jose, California, are the first of a family eventually to be made at ES2's own complex at Aix-en-Provence in the south of France.

The group, established with an initial capital of \$55 million, is headed by M. Jean-Luc Grand-Clement together with some of Europe's leading names in microelectronics design, including the ex-chairman of Britain's largest computer company, ICL. ES2 now boasts of 130 semiconductor and business experts forming the core of the group.

The objective is to encourage the more extensive use of silicon to solve microelectronic problems, or "to give designs in silicon a wider audience". The group plans to offer designers the forms of microchip circuits best suited to their needs, even when only relatively small numbers are required and when the interval between conception and manufacture must be short.

The group intends to develop a series of joint ventures across Europe, extending the pattern of collaboration begun by the two European projects ESPRIT (the European Commission's collaborative programme in information technology) and EUREKA (the series of collaborations in technology initiated by President Mitterrand of France).

Much of ES2's software, which is crucial to the success of the venture, is to be developed by British computer scientists, probably at the group's British base at Bracknell in Berkshire. The group is hoping that its turnover by the end of the decade will be over \$100 million a year.

Meanwhile, another European microchip venture, based in Britain, but with a US arm in Colorado, has launched a new microelectronic signal processor. This is the second major development from Inmos, now a subsidiary of the consumer electronics company Thorn-EMI. Since its launch in the late 1970s with British government funds, Inmos has been peppered with trouble. Largely the inspiration of the last Labour government, it was an attempt to give Britain a more significant presence in microchip manufac-

turing; it benefited from loans and grants before its sale to Thorn-EMI for £95 million two years ago.

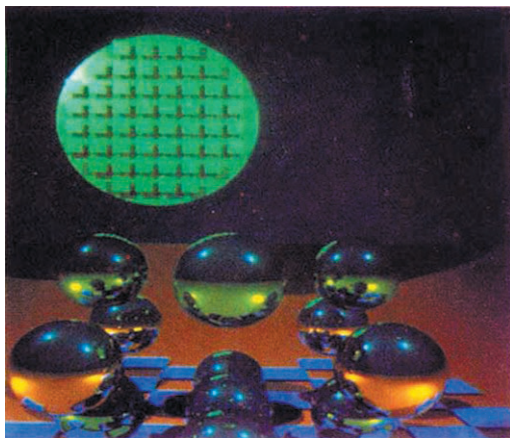
The mainstay of the Inmos microchip innovation is the transputer — a microchip that marries microprocessor and memory on one device. Launched almost a year ago, the transputer claimed 5 per cent of the 32-bit (input channels) microchip market in its first three months, giving Inmos an annual growth rate of 20–25 per cent, in the face of direct competition from the US semiconductor manufacturers Intel and Motorola.

Inmos's new signal-processing microchip has been designed to attract military and civilian users. For example, phase-shift analysis of signals allows accurate determination of the position of an object without the need to engage in conventional radar scanning. This microchip design is expected to be particularly relevant to sonar and in modems, where coding and decoding of information is required.

But the transputer will still be the company's mainstay. The next version of the chip, called the T800 and due to be launched next year, has been designed to improve floating point arithmetic, graphics applications and the speed of calculations,

providing a basic tool for intelligent workstations. Inmos has 150 or so people working on the development of the transputer at its Bristol base, together with a further 350 staff in Colorado. Its microchips are manufactured at Newport in Wales which employs a further 650 people.

A partnership between the researchers at the British Ministry of Defence and the electronics groups Ferranti and Marconi is another sign of the new assault on the



Computer graphics generated by the Inmos transputer.

international microchip market. What is claimed to be the world's first demonstrably fault-free microprocessor, capable of reliable performance in any environment, was unveiled earlier this month by researchers from the Royal Signals and Radar Establishment, the Ministry of Defence's complex at Malvern.

Bill Johnstone

Refusniks

Pressure easing in Soviet Union

THE long campaign of Dr David Goldfarb, the Jewish biochemist from Moscow, to leave the Soviet Union, is over. He arrived in New York two weeks ago, for urgent medical treatment after a more than usually bizarre campaign. During the past two and a half years, he reports, he has been granted an emigration visa and had it rescinded, turned down a proposal from the KGB to compromise the journalist Nicholas Daniloff (who kept him supplied with genetically engineered insulin unobtainable in the Soviet Union) and has been accused of trying to smuggle security-related material out of the Soviet Union (the material consisted of bacteriological strains of Western origin).

According to Dr Alexander Goldfarb of Columbia University in New York, the decision to allow his father to emigrate owed much to constant pressure from the scientific community. A few weeks ago, he said, six Nobel laureates (Andre Lwoff, François Jacob, Aaron Klug, Max Perutz, Francis Crick and Renato Dulbecco) sent a telegram jointly to Mr Eduard Shevardnadze and Mr George Shultz appealing for David Goldfarb to be allo-

wed to leave the Soviet Union.

The Soviet authorities seem prepared at present to make concessions on the issue of Jewish refusniks. In September a refusnik mathematician, Dr Mark Freidlin, was allowed to read a paper at an international symposium on statistics in Tashkent.

This relaxation, however, has posed ethical problems in Western scientists visiting the Soviet Union, some of whom have been urged by Soviet colleagues not to press the case of the refusniks too far. Liberal-minded Soviet scientists, it is suggested, put themselves at some risk by supporting the admission of Freidlin to the Tashkent symposium, and it would be unfair of Westerners to embarrass them by insisting on visiting other refusniks in Moscow or Leningrad.

Freidlin himself, however, is anxious that his own brief return to establishment academic life in Tashkent should not be made the occasion for depriving his fellow refusnik scientists of the visits from Western colleagues on which they depend to help preserve some kind of intellectual activity while waiting for an exit visa.

Vera Rich

Biowaste

Lightening a donkey's burden

A EUROPEAN Commission plan to use donkeys assisted by helium balloons, which would allow them to carry greater weights of biowaste down the mountains of the Abruzzo region near Rome — waste that would be converted to 'bunker oil' in small pyrolytic converters — has caused palpitations among some careful biotechnologists in the Commission, who fear it has threatened their modest but important attempt to move European chemical industries towards the use of agricultural feedstocks rather than coal or oil.

The Commission's "Concertation Unit for Biotechnology" (CUBE) has long argued the economic case against biofuels, and for some years has been energetically but soberly pursuing the development of biotechnology and its applications in Europe. But two weeks ago, CUBE members had heard nothing of the Abruzzo proposals, which had emerged largely through reports in the British press.

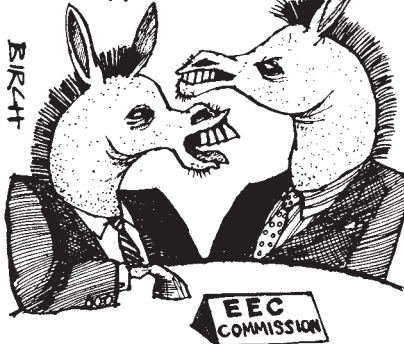
CUBE officials were privately furious that the Commission had been effectively (though unintentionally) smeared by connection with some of the more exaggerated aspects of the Abruzzo scheme, which began as the project of a small Italian entrepreneurial company, Italenergie, and has already been promised a pilot 46,000 million lira (£23 million) from the Italian regional authorities. The project's proponents in the Commission, who appear to have been taken in by an energy consultant's tongue-in-cheek remark that the transport of the 320,000 tonnes of waste a year envisaged in the full £150-million, 5-7-year scheme would be aided if the local donkeys were attached to balloons, are more sanguine about the proposal than are the CUBE people. Balloons aside, they aim to raise 33,000 million lira from the Commission's agricultural, development and social funds in support of the scheme, which would therefore not touch the Commission's research budget. These funds would buy 11 modular pyrolytic units each to convert one tonne of biowaste per hour to a kind of fuel oil with about half the calorific value per unit volume of ordinary oil.

Working with material from woods, wastelands and hilly regions, some 6 per cent of the energy requirements of the Abruzzo region could be met with the 30-40 pyrolytic converters of the scheme, together with a 27-megawatt electric power station (with district heating using waste heat) fired by the biofuel at a cost of around \$22 a barrel — admittedly dear at today's oil prices.

If the scheme — or the donkeys — get off the ground, biowaste may be extended by the addition of energy crops for marginal

land, such as yellow broom. This needs no fertilizer or irrigation, is insensitive to soil pH, and can be cropped at 10 tonnes per hectare per year, say supporters. In the longer term sweet sorghum on good soils could yield 14 tonnes of bioethanol per hectare per year, compared with the

So much for that mad idea—
now, back to the Butter
Mountain...



2.5-5 tonnes at present available from sugar beet. CUBE, the European Parliament and others have rejected bioethanol, a potential additive for transport fuel, as

uneconomic at present production costs based on sugar beet.

With its feet firmly planted on the ground, however, CUBE has been promoting something quite different: research to close the gaps between the chemistry of certain agricultural products, such as starch, and the carbon chains and other inputs required by the chemical industries. "We want a modest start to something of real strategic significance", said a CUBE spokesman. He did not want to "knock" the Abruzzo project, "which probably has real local and regional significance" (among other things it would create 2,000 jobs). He added that it was unfortunate that the fanciful flying donkey had emerged just as the CUBE "agroindustrial" project was approaching critical funding decisions by European research ministers.

But, it emerged last week that the prospects of EEC backing for the CUBE proposals had diminished. A European research ministers' meeting classified the project as "controversial". But, according to officials in Britain currently presiding over the meeting, the donkeys had had no influence on the downgrading. Matters such as funding levels in tight budgets and demarcation disputes between ministries of agriculture and industry were more to blame.

Robert Walgate

Italy to have research ministry at last

Milan

A NEW government bill is to be placed before the Italian parliament within the next year establishing a Ministry of Research and Technology. The bill will appear in the wake of legislation now making its way through the Italian legislature reorganizing CNR, the Italian National Research Council.

The reorganization has taken nearly 25 years to evolve, a product of much discussion between political parties, unions and research institutes, and represents a new commitment to research and development in Italy.

Italy has been slowly increasing the resources devoted to research in its attempt to reach a goal of 2.5 per cent of gross national product (GNP). The research budget from government coffers is still about 1 per cent short of that goal, although it has increased by 20.8 per cent (14 per cent in real terms) since 1985. The private sector and the public companies will share half of a budget which now totals 11,000 million million lire.

The new legislation represents a commitment by the Italians to improve the facilities and resources available to science. There are already 112,884 full-time staff in the research institutes of whom about half are engaged directly in research, making the average cost per researcher in Italy

about 130 million lire, roughly comparable to that in other developed countries.

According to Senator Luigi Granelli, the Italian Minister of Research, this is a very important time for the country's research and with more resources and reorganization, much more efficiency can be achieved. Based on the *Science Citation Index*, scientific productivity in Italy has improved substantially since 1983, lifting it from twelfth to eighth place last year.

That improvement will continue as a result of three principal measures. First, a commitment of an additional 1,500 million million lire over a three-year period for national research programmes involving research institutions and private industry, second, better industrial research with 100 million million lire a year over ten years through credits to industry from the Istituto Mobiliare Italiano (IMI) for applied research; and a national programme for research in biotechnology to start next year costing 400 million million lire.

The national funds for CNR will also be increased in the next twelve months to about 900 million million lire. Furthermore, an additional 720 million million lire in five years will be added to a 300 million million lire fund from industry, earmarked to improve the quality of researchers through 635 educational and training programmes.

Paola De Paoli

Bioregulation

Technical slip almost forgiven

Washington

A NATIONAL Institutes of Health (NIH) committee is recommending what hardly amounts to a slap on the wrist for Saul Kit of Baylor College of Medicine for failing to seek prior approval from his Institutional Biosafety Committee before field-testing a new vaccine made by recombinant DNA techniques. The committee also concluded that NIH guidelines need improvement to make clear when a new organism is regulated as a recombinant DNA organism, and what constitutes a deliberate release into the environment.

Early in 1984, an outbreak of pseudorabies, a potentially lethal disease in pigs, occurred on the Maddox farm in Texas. With Stewart McConnell, a veterinarian at Texas A&M University, Kit devised a plan to vaccinate the pigs on the farm. Kit and McConnell had the approval of the Texas Animal Health Commission. But they never sought NIH approval even though recombinant DNA technology was used in creating the vaccine (*Nature* 321, 190; 1986).

At issue is whether the vaccine contained recombinant DNA. Recombinant plasmids were used to produce the virus, but the final step yielded a virus made up only of pseudorabies virus DNA with a 148-base-pair deletion. The missing segment codes for thymidine kinase, thought to facilitate entry of the virus into

the nervous system of infected animals. The NIH committee also attempted to determine whether testing the vaccine in pigs was a "deliberate release into the environment".

NIH exempts from its guidelines recombinant DNA molecules that "consist entirely of DNA segments from a single non-chromosomal or viral DNA source". But this exemption is nullified if a protocol involves the deliberate release of an organism containing recombinant DNA. Kit maintains that the field test did not constitute an environmental release because the farm was quarantined, the vaccine was apathogenic, and it neither multiplied nor spread from vaccinated pigs.

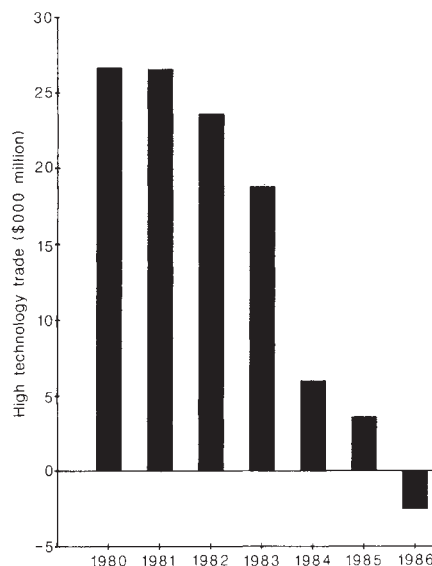
The NIH committee concluded that ambiguities in the NIH guidelines made it unclear whether Kit's action constituted a violation, but that "an experienced scientist" like Kit should have consulted his Institutional Biosafety Committee before proceeding with the field test. The committee recommended that Kit be forced to present plans for all future projects involving animals for a similar vetting procedure, something Kit says he would do in any case. The committee also recommended that NIH clarify their guidelines to remove ambiguities. At the last meeting of the NIH Recombinant DNA Advisory Committee (RAC), new language for the guidelines was proposed specifically to exempt organisms with deletions like

Congress hears of high-tech trade deficit

Washington

THE United States is facing its first ever deficit in high technology trade, according to a report* prepared for the Joint Economic Committee of the United States Congress. Although US trade has shown a net deficit since 1982, trade in computers, scientific instruments, aircraft and specialty chemicals has produced surpluses until this year. For the first half of 1986, high technology imports exceeded exports by \$1,300 million.

The report suggests several reasons for declining trade. An overvalued dollar makes it difficult for US companies to compete in foreign markets. Improvements in the US economy also spurred domestic demand, pulling in imports. But these factors are only part of the explanation. Decisions by other countries to support and protect domestic high technology industries have hurt US competitiveness. Export controls have also put a damper on US ability to exploit overseas markets. Finally the practice of granting "offsets", commercial *quid pro quo* for the purchase of US military hardware, appears to be having



an increasingly important impact on high technology trade.

Joseph Palca

*The US trade position in high technology: 1980-1986.

Immunology in India

New Delhi

THE National Institute of Immunology in New Delhi was opened by the Prime Minister of India, Mr Rajiv Gandhi, on 6 October. He commended the contribution that immunology can make to solving the country's problems in health care, birth control (see *Nature* 323, 661; 1986) and veterinary practice, and emphasized that applied science in India should not depend solely on basic research in the West. Other speakers, including the Prime Minister's science adviser M.G.K. Menon, stressed that linkage between basic and applied science is both a moral and a practical necessity.

The institute is housed in magnificent new buildings that include palatial animal facilities, a large laboratory block, auditoria and staff housing.

As well as birth-control vaccines, including gamete and trophoblast projects, work at the institute includes leprosy vaccine development, immunodiagnostics and basic work in reproductive immunology using transgenic mice. The director, G.P. Talwar, has been able to tempt back good young Indian scientists from abroad. The institute will help to disseminate the revolutionary impact of molecular biology on immunology.

Avrion Mitchison

pseudorabies vaccine.

Kit feels the NIH committee recommendations are a vindication of his position. Kit says he never submitted his plans for the Maddox farm test to the Baylor committee because he believed there was no question of violating NIH guidelines.

Joseph Palca

● The Biotechnology Science Coordinating Committee, which coordinates federal agencies' policies on recombinant DNA issues, has abandoned research guidelines proposed earlier this year by the US Department of Agriculture (USDA). Many of the comments received by the committee in response to publication of its "coordinated framework" for regulating biotechnology were particularly critical of the USDA guidelines, because they were different from and possibly inconsistent with those of NIH.

The USDA guidelines were published as a contribution to the "coordinated framework" (*Federal Register*, 26 June, p.23352) to address specifically the needs of agricultural research. But the plan now, according to Dr David Kingsbury, chairman of the coordinating committee, is to develop a unified set of research guidelines to apply to all federally funded research. The new guidelines would be based largely on those of RAC, with expansion of the provisions for environmental releases of genetically altered organisms. Proposals to regulate cell fusion and recombinant RNA would be dropped.

Tim Beardsley

European space

Manned spacecraft progress

EUROPEAN plans to build manned spacecraft advanced a little further at last week's meeting of the European Space Agency (ESA) in Paris, with the British scheme for a vehicle called HOTOL gaining a little on its French rival Hermes. West Germany's newer project, called "Sänger", which closely resembles HOTOL, also made a little progress.

In the corridors last week, there was talk that Hermes might be set back by pressure to provide this mini-shuttle with some form of escape system to guard against a disaster of the kind that overtook the US Challenger last January. The snag is that Hermes is planned to be only half the size of the US shuttle, so that the addition of escape systems may not be feasible. One possibility would be to restrict flights to military personnel, where the risk of the loss of life would be more easily justified. Even so, and in spite of doubts of the wisdom of spending so much on manned spaceflight, ESA agreed to a six-month technical study of the Hermes proposal.

The agency also agreed to support a three-year study of HOTOL and Sänger. Both projects involve horizontal takeoff. The West German project would lift a rocket-engined vehicle piggy-back through the lower atmosphere by means of a Mach-6 aircraft. The British project, technically more ambitious, would use a still-secret Rolls-Royce engine concept able to switch from an air-breathing to a rocket mode. Both projects are claimed to be capable of reducing launching costs to a fifth.

ESA's decision to study the two projects is a victory for British Aerospace, HOTOL's designer, which has championed the cause for the past two years. It also marks European concern that the United States and Japan will corner the world launch market in coming decades.

Hermes and the two horizontal launching vehicles are not technically competitive; Hermes is hardly more than an unpowered reentry vehicle whose success would depend on the development of later versions of the Ariane launcher. But there will be competition between the projects for funds, with Hermes priced (by France) at about £2,000 million to 1995 and HOTOL-like vehicles costing about the same but spread over two decades.

Hard choices are being put off until a ministerial meeting of ESA planned for the middle of next year, but last week's agreement to launch a "preparatory study" of Hermes, to be carried out at the Toulouse headquarters of the French national space agency, should show the way the wind is blowing. One sign will be the extent to which ESA members commit themselves to the cost of the planning

phase, estimated at 48 million ECU (£35 million). British officials were talking last week of the possibility of a 10 per cent contribution (twice the British stake in Ariane). A previously divided West German government has also indicated serious interest, although the final figures will not be known until the closing date on 30 November.

In Britain, the timescale is probably even shorter. The newly formed National Space Centre put a comprehensive plan for British research and development to

the government early in the summer. The government's response is expected in mid-November, before a final decision on Hermes must be made.

One question to be resolved is that present British spending on space, roughly £100 million a year, may have to be increased by roughly £50 million a year if the next ministerial meeting of ESA agrees to a 70 per cent increase of the agency's budget to cover, among other things, the cost of the production phase of Ariane 5. Full 10 per cent participation in the construction of Hermes, whose estimated cost some think could be a factor of five too small, would add another £20 million to the bill.

Robert Walgate

European research

Prospect of an empty barrel

EUROPEAN research ministers failed to agree last week whether to double the European Commission's research budget for 1987-91 — or to reduce it. The differences among ministers at an 'informal' lunch in Luxembourg were so great, indeed, that some in the Commission fear there will be no framework for research next year. Stop-gap proposals have been prepared for four individual programmes due to run out of cash in December (in telecommunications, health, aid to developing countries and aids to innovation in Europe).

Mr Geoffrey Pattie, Britain's technology minister, who is now president of the council of research ministers, is organizing a series of meetings in an attempt to reach a compromise before 9 December, the date of the last research ministers' meeting of the year and the last of his presidency.

The gap Pattie has to bridge is vast, that between the £2,500 million or so that Britain, France and West Germany believe would be in their interests and the £6,200 million proposed by the Commission and backed by most other states. Commission sources estimate a figure of around £4,200 million would be needed to maintain present support. Earlier this year it had been planning on a figure at least double this. It is clear that the figures now being discussed represent a major blow to Brussels' ambitions.

Brussels research commissioner Karl-Heinz Narjes is nevertheless said to be optimistic. He believes Pattie is under pressure to reach an agreement by December, and is also encouraged by the majorities by which most of the individual Commission proposals are accepted.

But majorities are not enough. Although most individual research proposals by the Commission are approved by a majority of governments, none may be agreed on this occasion because the Commission has followed the letter of the law and demanded unanimous agreement of

its package before discussing details. Although it is now technically possible for the Commission to sponsor programmes in which only some members participate, and even to allow outside participants, the Commission has in fact proposed no clear mechanism by which such programmes will be funded and agreed.

In fear that the framework will not be agreed, therefore, the Commission has proposed extensions to its RACE (Research and Development in Advanced Communications Technologies in Europe), medical health (largely AIDS and cancer), science and technology for development and SPRINT (Strategic Programme for Innovation and Technology Transfer) projects, all of which run out of cash in January. Of these, RACE is by far the biggest (£560 million over five years); it aims at developing European standards and common technologies in integrated broad-band communication networks. West Germany is firmly against RACE, however, being unwilling to open up its national telecommunications markets, and believing that its major electronics company Siemens can manage without outside collaboration. The medical health programme, it is claimed in Brussels, would link one in four of Europe's medical research groups in joint projects for a cost of £26 million over three years. In research for poor countries, a four-year £56-million programme would concentrate on improving the indigenous research capabilities in wide areas of agricultural and health research. SPRINT (£7 million over two years) would continue, among other things, the development of a network of management and technology consultants specialized in dealing with small and medium-sized companies, to help the innovation process cross national boundaries in Europe. Without funding in December, all these programmes will come to an end, the Commission says.

Robert Walgate

Time for divine intervention

SIR—R.J. Berry¹ reminds us that he and thirteen other professors of science in British universities “gladly accept the virgin birth, the Gospel miracles and the resurrection of Christ as historical events”. He insists, however, that our standards of evidence in approaching apparent miracles should be “just as rigorous” as those we employ for any scientific observation. It is therefore appropriate to note that the virgin birth is documented within the New Testament only in the two nativity stories of Matthew and Luke, and that theologians have long admitted that each is full of its own difficulties, as well as being incompatible with the other. Kümmel says in his standard handbook that they “contradict each other in essential features”².

On the Catholic side, R.E. Brown’s full-scale study admits that it is “quite impossible” to harmonize them, and that we have “no reliable information about the source of either”³. Mark, the earliest evangelist, introduces Jesus as an adult and records nothing of his early life. Paul, writing still earlier, says that Jesus was “born of a woman” (Galatians 4:4) and this does not suggest acquaintance with the doctrine. Even so conservative a scholar as C.H. Dodd attempted only a half-hearted defence, calling the two infancy narratives a “structure of imagery” with “a basis in fact somewhere behind it all”⁴.

As to the resurrection, the gospel narratives contradict each other in essentials, as when one evangelist makes Jesus’s appearances to his disciples occur exclusively in Galilee, while another sites them exclusively 80 miles away at Jerusalem. Paul knows nothing of the place of crucifixion, let alone of resurrection. Even the nature of the post-resurrection appearances is not the same in the various accounts. Did Jesus return for a time to normal life with his disciples, or appear only from on high with a body of heavenly radiance? The theologian D.F. Strauss was perfectly justified in saying of the resurrection: “Rarely has an incredible fact been worse attested, and never has a badly attested one been intrinsically less credible”⁵. Dodd allowed that “the historian may properly suspend judgement” as to whether “Jesus had in some way left his tomb”⁶. Such concessions have come to be made only because vindicating the actuality of the event on the basis of the New Testament evidence has proved a well-nigh hopeless task.

That such divergent accounts could be written by authors who had already come to believe (for reasons that need to be investigated) that Jesus was virgin-born and rose from the dead is perfectly plausible: that their narratives provide any basis for such belief is not. The events alleged in these stories did not form the basis of the

faith, but the stories resulted from faith. One suspects that the professors of science who accept the miracles recorded in these and other gospel stories are less well acquainted with the inadequacy of the documentary evidence than are many of their theological colleagues.

G.A. WELLS

*Department of German,
Birkbeck College,
23 Gordon Square,
London WC1H 0PD, UK*

1. *Nature* 322, 321 (1986).
2. Kümmel, W.G. *Introduction to the New Testament* (English translation) 43 (SCM, London, 1975).
3. Brown, R.E. *The Birth of the Messiah* 7 & 497 (London, 1977).
4. Dodd, C.H. *The Founder of Christianity*, 31 (Collins, London, 1971).
5. Strauss, D.F. *Der alte und der neue Glaube*, 72 (Leipzig, 1872).
6. Dodd, C.H. *The Founder of Christianity*, 166–167 (Collins, London, 1971).

SIR—It is hard to disagree with R.J. Berry’s discussion of the difficulties inherent in the scientific verification or repudiation of “miracles”¹, which by their unique nature appear necessarily opaque to the scientific method. Matters of faith aside, even the personal experience of a miraculous event would not meet the standards of scientific publication. It is also difficult to disagree with his comment that “science has limits”, as modern philosophers opposed to the demythicization of previous cultures have suggested^{2,3}.

Our success as scientists does not compel the belief that science is sufficient to encompass all possible aspects of life, since the scientific method, powerful as it is, is not supplied to us *a priori* (in which case it would be a “miracle” itself). Certainly such a relativistic attitude need not detract from our ability to perform scientific work.

JAMES A. SCOTT

*Massachusetts General Hospital,
Boston, Massachusetts 02114, USA*

1. Berry, R.J. *Nature* 322, 321 (1986).
2. Hubner, K. *Critique of Scientific Reason* (University of Chicago Press, 1983).
3. Eliade, M. *Ordeal by Labyrinth* (University of Chicago Press, 1982).

SIR—On the subject of My Divine Essence, Character, Attributes, Inclinations, Motives, Philosophy, Politics and *modus operandi*, especially as regards what is vulgarly referred to as *miracles*, speculations about which have recently been appearing in this magazine, and even more especially as regards My hypothesized role in Universe construction where, so it has been alleged, I, in the form of a bipedal primate outwardly resembling an elderly and bearded male *Homo sapiens sapiens*, created the Universe for the amusement and edification of men, I should like to state:

(1) I am not a very good Member of the Church of England. (2) When I come to think of it, I am not even a bad Member of the Church of England. (3) When I really pause to consider the matter, I fear (if that is the word) I am not even a Christian, having been around an eternity before that particular creed was organized, and consequently, am Supremely indifferent to its beliefs, dogmas and convictions.

I am, therefore, rather inclined to take a dim view of *all* people of whatever persuasion voicing opinions on Me, this for the simple reason that, as I am Ineffable and exist beyond all human comprehension in no particular spatio-temporal relation to the physical cosmos, *all* opinions about Me are meaningless, when not actually insulting, and, what is worse, tiresomely predictable, especially to One Who knows all things, reflecting as they do only the outlooks and attitudes of these, My unsolicited and intellectually and imaginatively limited interpreters who, if ever they *really* thought about Me, would be more inclined to keep silent.

You will understand that, while I am not in the habit of presenting My views on theology in the correspondence columns of periodicals (except as occasionally in the past to *The Times*), I am taking this opportunity to do so because, though as is well attested, My Patience is infinite, It is not *that* infinite. Nor, may I remind your contributors, does being Omniscient and Omnipotent of necessity compel One to be All-Benevolent.

I in parting, hope that this correspondence can now be closed. GOD

(As revealed to Ralph Estling)

*The Old Parsonage, Dowlish Wake,
Ilminster, Somerset TA19 0NY, UK*

● God’s will be done; this correspondence is now closed. Editor, *Nature*.

Student loans

SIR—In response to your leading article entitled “Social security for students” (*Nature* 321, 797; 1986), I would remind you of a scheme once proposed for the United States by the late Professor Jerrold R. Zacharias: that students receive adequate maintenance grants from the central government against a lifelong repayment scheme in the form of a surtax upon their annual income tax. Those who are financially successful would eventually reimburse more (or even much more) than they receive; those who earn less (including ministers, teachers, university lecturers and others in socially valuable but impecunious professions) would be automatically forgiven a part of their grants.

In equilibrium, the surtax should amply finance current grants; the major obstacle is how to finance the starting transient.

BEROL ROBINSON

*1 Rue du Général Gouraud,
92910 Meudon, France*

New ways with classical mechanics

For about a century, the chief defect of classical mechanics has been that most problems within its ken have been insoluble. Now there is some hope that complex systems will be tractable.

THE optimism of nineteenth-century physics in its belief that all problems could be solved by Newton's laws and their later elaboration is now well recognized and widely scorned, but not for all of the right reasons. The nineteenth century was justly proud of the sophistication of schemes such as hamiltonian mechanics, which offered the solution of all problems provided that the arithmetic (or algebra, or calculus) was feasible.

The usual complaint is that the optimists overlooked the threat to the foundations of mechanics embodied first by Maxwell's theory of electromagnetism and, later, by even more subversive developments. More recently, people have also been saying that it was foolish of the nineteenth century to suppose that the arithmetic of the solution of real problems, with numbers of variables of the order of 10^{23} , would be simply a procedural matter. But now, almost a century after the full flowering of nineteenth-century optimism, the question arises whether the critics might not themselves have used the interval more constructively.

Classical mechanics is fine so far as it goes, but that is not very far. Only the simplest problems are soluble. For the rest, it is possible to extract from, for example, the hamiltonian formalism a few 'constants of the motion' which may, in special circumstances, be physically relevant. But what use can it be, for example, in an attempt to calculate the Curie temperature above which a ferromagnetic material cannot be permanently magnetized, to know that the physical linear and angular momenta of the specimen will be conserved? The quantities that naturally arise in nineteenth-century mechanics are often not particularly interesting.

That is why there has recently been such interest in other ways of calculating the physical properties of even classical complex systems. The twentieth century's shame is that most of these schemes are only recent. One landmark is the exact calculation, by Onsager in the 1940s, of the properties of a two-dimensional Ising lattice, most simply a square lattice whose vertices are of two kinds and in which only pairs of nearest neighbours interact. This can serve both as a model for order-disorder in alloys such as β -brass and for ferromagnetism. Another closely related approach to the calculation of complex systems is that represented by cellular automata, which may be likened to living

organisms that replicate themselves from one generation to the next by means of formal rules.

The objection to most of these methods is that they tend to require numerical simulation, so that general principles can be discovered only with difficulty and enormous labour. One of the most familiar illustrations is the use of T.A. Witten's model of the process of aggregation for studying the formation of aggregates in circumstances where the process is limited by the rate of diffusion (Witten, T.A. & Sander, L.M. *Phys. Rev. Lett.* **47**, 1400; 1984); the proof that aggregates are fractal structures has nevertheless helped to develop scaling laws relating the total mass of an aggregate to its linear dimensions, for example.

Two other kinds of problems conventionally tackled numerically have now been given more general treatment, suggesting that people's ambitions are now rising to the point at which it may be feasible to ask interesting questions of some simple models of complex behaviour. One such model is that of the process of diffusion on a tree-structured lattice, which is in principle a model for all kinds of systems. If the 'leaves' of a tree-shaped graph are the blind end-points most distant from the root, for example, it is a fair question to ask how an arbitrary distribution of some attribute among the leaves will rearrange itself statistically if particles, or attributes, can move from one leaf to another only by the branches of the underlying tree.

Constantin P. Bachas and B.A. Huberman rightly note, in an article just published, that the rearrangement of some attribute among the leaves of a tree is a process representative of the behaviour of systems organized on hierarchical lines (*Phys. Rev. Lett.* **57**, 1965; 1986). In this spirit, for example, one might ask how the microscopic properties of protein molecules are determined by the hierarchy of amino-acid sequence and domain structure. The particular interest of what they have now accomplished is what seems like an analytical solution of the problem of diffusion on a tree-shaped graph even if the underlying structure (for example, the number of branches at each vertex) is far from simple.

Bachas and Huberman work with the concept of the redistribution of probability among the leaves of a tree, but the idea is more generally applicable. One of their

conclusions is that the process of relaxation, or rearrangement towards a stable configuration, is most rapid for fat than for thin trees, which may not in itself be surprising; several branches at each vertex will give both a more bushy tree and more opportunities for lateral transport. It is more surprising that they conclude that diffusion is inherently more rapid for uniform trees (with a fixed number of branches at each vertex) and for random trees than for all intermediate structures.

The succeeding article in the same issue of the same journal is a step in the same direction by Martin Grant and J.D. Gunton of Temple University in Philadelphia. What they have done is to construct a continuum analogue of the cellular automaton construction — and to solve it (*ibid.*, p.1970). The essence of the problem is that, unlike the microscopic dynamics of the nineteenth century, this problem is irreversible in time. There is an obvious connection with irreversible dynamics, where the transformation of the state of a mechanical system from one instant to the next is represented by a matrix which is not unitary (or hermitian). These are also of course the systems in which people such as Ilya Prigogine have argued that highly organized systems (such as living things) may emerge not merely as accidents but because they are enforced by the underlying dynamics.

Grant and Gunton give an elegant account of how it may be possible to isolate from all the variables of a complex system those which vary only slowly (compared with others) in time, and of how dissipation can force the 'slow' variables to grow exponentially with time, at least at the beginning of the evolution of a system. The argument makes believable the observation, in numerical simulations of cellular automata, of recurring apparently well ordered patterns. The authors refer in passing to the familiar rapid growth of dendrites in crystallizing systems; these are structures far from ordinary thermodynamic equilibrium that appear to be forced by the underlying physics. Whether Grant and Gunton's conjecture of "irreversibility leading to [thermodynamic] instability" will be found valid, and whether it will, for example, account for the existence of self-organizing systems such as living things, is for the time being an open question. But it is an important question, far removed from hamiltonian dynamics.

John Maddox

Neurobiology

Making of the nervous system

from R. W. Guillery and C. D. Stern

MANY conferences in the growing field of developmental neurobiology produce a plethora of facts and figures with no clear sense of direction. In a relatively new field, where the neurobiologist can still be surprised by classical concepts of embryology and may be unaware of recent cell-biological advances based on non-neural cells, this is perhaps not surprising. A recent conference* succeeded in bringing together investigators with diverse views. Although molecular biologists, the newest recruits to neurobiology, were not represented, vertebrate and invertebrate neurobiologists and developmental biologists were able to exchange views. The meeting consisted mainly of reviews of the processes that contribute to shape the nervous system, from the linkage of individual cells to activity-sharpening and cell death. It was an opportunity for those dazzled by the new techniques of molecular biology and the proliferation of messenger RNAs expressed in one or another portion of the central nervous system to be reminded of some of the basic problems that remain to be solved.

Several of the speakers bridged the gap between traditional cell biology and neurobiology. For example, microtubule-associated proteins have quite distinct and characteristic distributions in nerve cells, some associated with distinct subpopulations of tubules and some not associated with tubules at all (A. Matus, Friedrich Miescher Institute). Even early in development, neural cells are as varied as non-neural cells: neural crest and neural placode derivatives differ in their requirements for specific growth factors (R.M. Lindsay, Sandoz).

Much of the discussion centred on the subject of the existence of public or axon-specific highways that may pave the way for groups of growing axons (such as glial guides; J. Silver, Case Western Reserve University) and of diffusible molecules that may signal the location of targets. Although no revolutionary new mechanisms were revealed, some important concepts were challenged. It seems that neither laminin nor fibronectin nor their receptors are distributed in a manner that is consistent with the migration pathways of neural crest cells or segmental nerves (M. Bronner-Fraser, University of California, Irvine), and that the adhesion molecule N-CAM is not involved in either neural induction or in the guidance of axons in *Xenopus*, although it is involved in axon fasciculation and is down-regu-

lated during the establishment of a retinotectal map (M. Jacobson, University of Utah). In an elegant search for specific markers on the surface of axonal processes, fasciculation of axons *in vitro* was found to be altered by some monoclonal antibodies but not by anti-N-CAM (J. Raper, Max Planck Institute, Tübingen). In some cases the target tissue can emit a target- and nerve-specific diffusible signal, capable of directing axonal growth, that propagates over relatively long distances *in vitro* (A.G.S. Lumsden, Guy's Hospital Medical School), and during the early stages of patterning of the peripheral nervous system, such as the early outgrowth of segmental nerves, it seems probable that inhibitory molecules prevent motor nerves and neural crest cells from entering into the wrong regions (R.J. Keynes, Cambridge University; C.D.S.).

The complexity of the vertebrate peripheral nervous system presents an aesthetically pleasing picture. Individual axons can now be differentially stained to show that some muscle fibres are always multiply-innervated whereas others replace their initial multiple innervation by a single innervation (J.W. Lichtman and D. Purves, Washington University, St Louis). Although the nervous systems of invertebrates may seem simple at first glance, detailed analysis shows that interactions between the developing parts are very complex both in insects (J.P. Bacon, University of Sussex; see the *News and Views* article on p.758) and in leeches (S. Blackshaw, University of Glasgow) and nematodes (R. Durbin, MRC Laboratory of Molecular Biology). The detailed

mechanisms may not always be the same as those that operate in vertebrates. In the absence of convenient vertebrate mutations such as *minute* in *Drosophila*, simply mapping cell lineages cannot give information about lineage restrictions or the existence of insect-like compartment boundaries in vertebrates (S.E. Fraser, University of California, Irvine).

In the vertebrate central nervous system it is difficult even to define the questions, as there is much information but little theory to explain how more complex patterns are generated. The retinotectal system is a classic challenge, providing employment and frustration for many developmental neurobiologists. The formation of an accurate map involves several distinct developmental strategies, which operate coordinately (J.T. Schmidt, State University of New York at Albany; C. Stuermer, Max Planck Institute, Tübingen; J.W. Fawcett, Salk Institute). The assumption that, in producing maps, layers, columns or architectonic borders, axons compete to establish connections is challenged by the argument that competition, like embryonic regulation, might be caused in many instances by experimental interference (R.W.G.). Indeed, the possibility that by challenging a system experimentally and forcing it to perform under extreme circumstances one can start to elucidate 'normal' development can be questioned in relation to almost any problem in developmental biology. The criticism can be met only by devising sufficiently subtle and varied approaches . . . "so much concerning the several classes of Idols, and their equipage: all of which must be renounced and put away with a fixed and solemn determination, and the understanding thoroughly freed and cleansed" (Francis Bacon). □

R.W. Guillery and C.D. Stern are in the Department of Human Anatomy, University of Oxford, OX1 3QX, UK.

Low-temperature physics

Pauli principle in a gas

from Peter McClintock

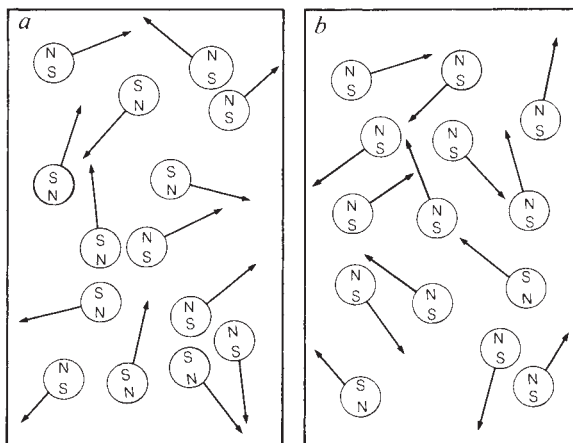
A NEW and macroscopic demonstration of the Pauli exclusion principle, not in the familiar quantum liquids but in a gas, has been proposed by J.P. Bouchard and C. Lhuillier. Writing in a recent issue of *Physics Letters A* (116, 99; 1986), they suggest a series of experiments to seek an intriguing new kind of convective instability in dilute spin-polarized gases — that is, in gases whose constituent particles have magnetic moments mostly aligned in some preferred direction (see figure).

The Pauli principle is, of course, very familiar to anyone who has ever studied chemistry. By requiring that no two elec-

trons can ever be in exactly the same quantum state, it is directly responsible for the existence of the elements, each with its own distinct and stable electronic configuration. The Pauli principle is also of much wider applicability, however, in that it governs the behaviour of all collections of entities whose angular momenta, or spins, are half-integral. Such fermions include electrons, ³He atoms, neutrons and protons. It has long been known that collections of mobile fermions can display large-scale, or macroscopic, quantum effects in the low-temperature limit, where all the available quantum states are

*The Making of the Nervous System Wye College, Kent, UK. 8–12 July 1986.

Schematic diagram of a gas of ^3He atoms with a weak magnetic field pointing in the vertical direction. The atoms are moving in random thermal motion (arrows), and the orientations of their magnetic nuclei are shown by indicating the positions of their north (N) and south (S) poles. *a*, In an unpolarized gas, there are equal numbers of nuclei magnetized with and against the applied magnetic field. *b*, In a spin-polarized gas, most of the nuclei are magnetized in the same direction.



occupied up to a critical value (the Fermi energy) and all the states with larger energies are empty. Superconductivity of the electron gases in certain metals is a particularly dramatic example of this degeneracy. Bouchard and Lhuillier address, however, the rather different situation that pertains at somewhat higher temperatures, where the system is non-degenerate and the distribution of energies tends more towards its classical form.

Under such circumstances it is at first sight not obvious that the Pauli principle is going to be of any great significance because, by and large, at such temperatures so many states are available that no two particles are going to need to be in the same state anyway. Where the Pauli principle does exert an important influence, however, is in collisions between the particles — such collisions being of vital importance in determining the macroscopic properties of the gas. For example, two ^3He atoms that approach closely enough will repel each other, partly on account of the electrostatic repulsion of their respective electron clouds but also, if their spins are orientated in the same directions, because of the Pauli principle which tends to push the atoms apart to prevent their occupation of the same overall quantum state of motion.

In the case of the ^3He atoms, the net spin and corresponding magnetic moment are associated with the nucleus: 2 protons and 1 neutron, each of net spin $\frac{1}{2}$, leading to an overall spin of $\frac{1}{2}$. Two colliding ^3He atoms will behave quite differently, depending on whether their spins are parallel or opposed. Consequently, the spin-polarized gas, where the spins are fully polarized or preferentially aligned in some preferred direction, may be expected to have properties rather different from those of an ordinary gas composed of the same atoms in which the spin populations are equal. (The spin polarization of the sample is surprisingly long-lived because of the requirement of angular momentum conservation, despite the huge numbers of collisions that occur, and can be regarded as a valid parameter for use in characterizing the system.)

An interesting and unexpected consequence of these considerations is that the temperature and degree of polarization — normally thought of as quite independent variables — should become coupled. This thought leads Bouchard and Lhuillier to a fascinating experimental proposal. Because of the temperature — polarization connection, the rate at which heat is carried through a sample of spin-polarized gas should depend not only on the temperature gradient (as usual) but also, through a coupling coefficient L_{12} , on the spin (or magnetization) gradient. Similarly, the rate at which magnetization propagates should be dependent, through a coefficient L_{21} , on the temperature difference across the sample as well as on the magnetization difference across it.

Bouchard and Lhuillier's ingenious use of this circumstance involves setting up two quite different convective instabilities in opposition to each other. The first of these is the well-known Rayleigh-Bénard

instability: when a slab-shaped volume of liquid is heated from below, reducing the density below relative to that above, there will be a critical temperature difference between top and bottom at which it suddenly starts to convect under gravity. The second instability is a novel magnetic version of the Rayleigh-Bénard one. If there is a magnetization gradient in a fluid an adverse magnetic field gradient applied at the same time will tend to turn it upside down just as gravity does a fluid heated from below. But in a spin-polarized gas, a thermal gradient will also produce a magnetization gradient. So by proper choice of magnetic field, it is possible to set up the two instabilities, one gravitational and one magnetic, in opposition to each other. If the balance is right, the classical terms in the overall stability equation cancel out, leaving only the relatively much smaller Pauli principle terms. Under these circumstances, any consequent motion of the liquid — which would be most interesting to observe — would effectively represent a macroscopic manifestation of the Pauli principle. It would also provide a means by which L_{12} and L_{21} could be measured experimentally.

An experiment of this kind will not be particularly easy, but neither does it appear to be totally out of the question: the required densities and polarizations are not too greatly in excess of what can currently be achieved. It is to be hoped that somebody with the necessary skill (and courage) will bring it to fruition. □

Peter McClintock is in the Department of Physics, University of Lancaster, Lancaster LA1 4YB, UK.

Molecular biology

Genetics of filamentous fungi

from John Fincham

NOTWITHSTANDING the spectacular successes of yeast molecular biology, *Saccharomyces* is an odd organism in many ways and other fungi may be better general models for eukaryotes. This is one rationale for the current surge of interest in filamentous fungi; the hope of biotechnological benefit is certainly another. A recent workshop* brought together these academic and commercial interests. Here I discuss only the highly molecular end of a broad spectrum of contributions.

The most important recent breakthrough for fungal geneticists has been the development of reliable transformation with DNA. There have been improvements in transformation procedures for *Neurospora crassa* and *Aspergillus nidu-*

lans and successful transformation in a wide range of other species, representing all the main fungal groups. The following tentative generalizations are based mainly on *Neurospora* and *Aspergillus*. Fungal DNA sequences ligated into bacterial circular plasmids may sometimes be able to replicate in fungal nuclei, but as yet there is no plasmid construction capable of stable replication and transmission. This lack seems less important than it once did because a good degree of stability is usually achieved through integration of the transforming DNA into the chromosomes.

The integrated sequences are sometimes found inserted at their normal chromosomal loci, either in replacement of the homologous sequences of the recipient organism (attributed to gene conversion) or by insertion of the whole plasmid (as if by a single crossover). Frequently, howev-

*Molecular Biology of Filamentous Fungi EMBO workshop at the National Institute of Agronomy, Grignon, France 1–5 September 1986, organized by C. Scazzocchio and M. Picardo.

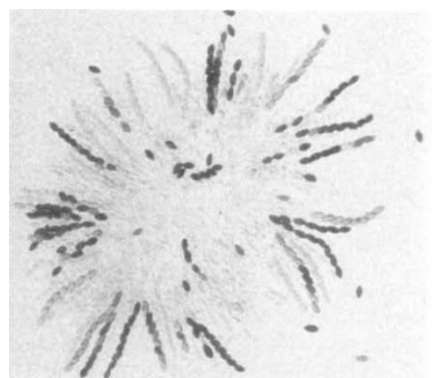
er, integration is non-homologous, without apparent locus specificity. The proportion of homologous to non-homologous integration varies widely from one gene to another, even within the same species (for example, *N. crassa*; A. Radford, University of Leeds). Nothing is known about the mechanism of and sequence requirements for non-homologous integration. Nor is it understood why integrated genes often seem to get lost or silenced during meiosis. One obvious possibility is that such instability is caused by recombination among tandemly repeated gene copies, some of them imperfect; if this is the explanation, single-copy integrations should be stable, a prediction not yet adequately tested.

Transformation opens the way to gene cloning (either by recovery of the transforming plasmid or by 'sib-selection' from pools of genomic clones), and cloning is the key to the study of gene regulation. M.J. Hynes (University of Melbourne) has cloned the *Aspergillus* structural gene encoding the enzyme acetamidase (*amdS*), and has located separate upstream binding regions for the products of four positively acting regulatory genes. Each of these regulatory genes controls the transcription of a set of enzyme-encoding genes, and *amdS* is a member of all four sets. One of the regulatory genes, *areA*, the central regulator of nitrogen catabolism, has been cloned (H.N. Arst, London Postgraduate Medical School) by a method which, for once, owes nothing to transformation and everything to a detailed knowledge of the formal genetics of the fungus. In another well-worked *Aspergillus* regulatory system (B. Felenbock and C. Scazzocchio, University of Paris at Orsay), *alcR* encodes a product that regulates positively both *alcA* (alcohol dehydrogenase) and *aldA* (acetaldehyde dehydrogenase). It also regulates *alcR* itself. All three genes have been cloned and most of their sequences are known. The study of DNA-protein interactions, not to mention chromatin structure, comes increasingly on to the agenda.

Impressive advances were also described in another area of gene regulation — the control of the mitotic cycle. The *wee-1* gene of *Schizosaccharomyces pombe* (fission yeast, smuggled into the meeting as a degenerate filamentous fungus) has been cloned; the protein that it encodes has some similarity with known protein kinases (P. Nurse, ICRF, London). This gene seems to act at two separate points in the mitotic cycle. In *A. nidulans*, *nima* has an essential function immediately before chromatin condensation (S. Osman, Rutgers University); it too has been cloned. Work is proceeding on the other components of the regulatory networks in which these genes act.

There is new information on the identification of unidentified reading frames in

the mitochondrial genomes of *Aspergillus* and *Neurospora* (T. Brown, UMIST; M.A. Nelson, University of Rome); some of these are now known to encode subunits of the NADH oxidase complex, functions which, in *Saccharomyces*, are the



N. crassa asci containing ascospores (products of meiosis) from a fruit body. $\times 700$.

responsibility of nuclear genes. Mechanisms of intron splicing in *Neurospora* are being elucidated through the use of *in vitro* systems and mutants blocked at different steps (G. Garriga, Massachusetts Institute of Technology). Considerable attention was given to the molecular pathology of the mitochondrial genome. A type of mycelial senescence in *Podospira anserina* (L. Belcour, Gif-sur-Yvette) is caused by proliferation of mitochondrial genomes that have suffered extensive deletions, rather as described already for *Saccharomyces*. But a quite different explanation applies to a

senescence-prone Hawaiian strain of *Neurospora intermedia* (H. Bertrand, Regina, Saskatchewan). This strain has a multicopy linear 9-kilobase plasmid in the nucleus which, in senescent cultures, becomes integrated into the mitochondrial DNA. It is uncertain whether this plasmid, called Kalilo (said to be Hawaiian for 'death's door'), ever integrates into the chromosomes. Its most curious property is its failure to be transmitted by the male parent through sexual crosses, which, in the absence of other evidence, would have been taken as excluding a nuclear location. Suitably modified to cure it of its propensity to invade the mitochondria, Kalilo might be useful as a cloning vector.

There is optimism about the industrial applications of filamentous fungal molecular biology, particularly in the light of what has already been achieved with *Aspergillus* spp. (W. Davies, Allelix, Ontario). If transformed with multiple copies of a construction containing an efficient inducible promoter (such as that of *alcA*) upstream of an open reading frame encoding a synthetic signal sequence fused in-frame to the coding sequence for the desired protein, the transformed mycelium can be induced to devote most of its energy to secreting the protein into the medium in a form relatively free of cellular contaminants. This sounds like good news for research on filamentous fungi. □

John Fincham is Professor of Genetics at the University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.

Insect pheromones

Beauty is in the antennae of the beholder

from Jonathan Bacon

ON the morning of 6 May 1875, the French naturalist Jean Henri Fabre placed a newly emerged female of Europe's biggest moth (the Great Peacock) into a wire-gauze cage. As night fell, his house was invaded by many amorous conspecific males. In his own words¹, "Coming from every direction and apprised I know not how, here are forty lovers eager to pay their respects to the marriageable bride born this morning amid the mysteries of my study".

We now know how male moths are apprised of the location of conspecific females. She has an abdominal gland that produces a pheromone and he has specialized antennal receptors to detect it. The incredible sensitivity of these receptors (allowing male moths to find a female at a range of miles) is legendary and warrants their mention in the *Guinness Book of Records*². This is a sex-specific behaviour;

the female has none of these antennal pheromone receptors, rendering her unresponsive to the female attractant. The paper by Schneiderman, Hildebrand and colleagues on page 801 of this issue³ describes the remarkable result that transplanting the male antenna to the female of *Manduca sexta*, the tobacco hornworm moth, causes her to exhibit male-specific behaviour — the female flies to a female pheromone source.

Holometabolous insects (those that go through a metamorphosis) such as moths lend themselves to this kind of experimentation. The larva carries within itself the adult body parts as undifferentiated modules called imaginal disks, infoldings of epidermal cells set aside during embryogenesis. Metamorphosis at the pupal stage is brought about by the growth, eversion and differentiation of these disks. As this is going on, the central ner-

vous system is drastically remodelled to mediate the change from the crawling, non-sexual behaviours of the caterpillar to the flying and sexual behaviours of the adult moth. To do this, some neurones die, some are remodelled and some develop *de novo*⁴. What emerges are animals that display the overt kind of sex-specific behaviour described above.

The antennal-disk epidermis gives rise to the antennal sensory neurones whose axons navigate to the antennal lobes of the central nervous system at the pupal stage. The lobes become organized into glomeruli which may be the targets of afferents responsive to different airborne molecules. In normal males, one of these glomeruli is particularly large and is the termination point of the male-specific afferents. This macroglomerulus is absent in normal females⁵. Because each antenna develops from its own disk, gynanders can be readily constructed by replacing female disks with male ones at the late larval stage⁶. This operation has a dramatic effect on the brain; the male sex-specific antennal sensory neurones, forced to grow into the foreign environment of the female brain, cause the appearance of a macroglomerulus and interneurones responsive to the female attractant⁶. These elegant anatomo-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Great Peacock moth (*Saturnia pyri*) — early observations (Bruce Coleman).

mical and physiological observations were limited to the brain; the paper in this issue³ provides the evidence that the cellular changes manifest themselves behaviourally, as females with male antennae show anemotaxis to the female pheromone. Interestingly, these experimental animals do not fly in circles as if confused by the presence of their own pheromone gland.

Females do have their own sex-specific behaviour which resembles the anemotaxis of the male in some respects; tobacco plants are the requisite sites for egg laying and females (but not males) show positive anemotaxis towards them. Work in progress in Hildebrand's laboratory will reveal whether males with female antennae show a sudden interest in tobacco.

One interpretation of the data presented in this issue³ might be that females have sex-specific antennal receptors tuned to some element of the cocktail of odours emanating from the tobacco plant. These tobacco receptors in the female and the pheromone receptors in the male would provide the sensory input to the same



DENSE clusters of the chemosynthetic tube worm *Lamellibrachia* sp. were discovered co-occurring with the bivalve *Acesta* sp. 130 km south of Louisiana at a depth of 635 m. The bivalves, which have unfused mantle margins and feed by filtering water past ciliated gills, are in all cases attached to the distal ends of the tube worms. This close association may have important implications for the physiology of the chemosynthetic *Lamellibrachia*. The photograph was taken by LGL Ecological Research Associates Inc., Texas, supported by the US Department of the Interior. Gregory S. Boland

anemotaxis motor circuits in the central nervous system in both normal and transplant animals. (The size of the male macroglomerulus would result from the large number of pheromone-sensitive afferents.)

A clear way to refute this notion would be to demonstrate that females bearing male antennae still show anemotaxis to tobacco plants. But even in the absence of this behavioural evidence, it seems unlikely that the female does have her own set of sex-specific afferents since both male and female brains contain 'generalist' interneurones that respond to tobacco odours (ref. 5; J.G. Hildebrand, personal communication). It seems more likely that it is the presence or absence of the male-specific afferents which influences the development of the central nervous system, with important behavioural consequences. The central issue, of course, is how does it work? In one scheme, interneurones of the antennal lobes would be pluripotent at the time of afferent ingrowth and the ingrowing male-specific afferents would provide some signal to cause the determination and differentiation of male-specific pheromone interneurones (instructive development). In another, the antennal-lobe interneurones may already be determined at the time of afferent arrival; only those cells receiving an appropriate signal survive (permissive development) — interneurones without the signal die.

In the moth, it is not easy to distinguish between these two possibilities at the cel-

lular level because the olfactory system comprises thousands of neurones. Large cell populations do, however, encourage other approaches. Hildebrand and colleagues are investigating the nature of the putative signal that triggers sexual differentiation by examining the biochemical and antigenic differences between male and female antennal afferents^{7,8}.

The mate-seeking behaviour that Fabre first observed more than 100 years ago is likely to provide a focus of research for years to come. The discovery that the sex of the antenna alone seems to be critical for the sexual differentiation of the brain and anemotaxis to a pheromone source provides a dramatic example of the common, yet poorly understood, mechanism by which cells affect the development of their neighbours. □

1. Fabre, J.H. *The Life of the Caterpillar* (McClelland, Goodchild and Stewart, Toronto, 1916).
2. McWhirter, N.D. (ed.) *Guinness Book of Records* (Guinness Superlatives, London, 1986).
3. Schneiderman, A.M., Hildebrand, J.G., Brennan, M.M. & Tumlinson, J.H. *Nature* **323**, 801 (1986).
4. Levine, R.B. & Truman, J.W. *Nature* **299**, 250 (1982).
5. Matsumoto, S.G. & Hildebrand, J.G. *Proc. R. Soc. B* **213**, 249 (1981).
6. Schneiderman, A.M., Matsumoto, S.M. & Hildebrand, J.G. *Nature* **298**, 844 (1982).
7. Kingham, T.G. & Hildebrand, J.G. *Soc. Neurosci. Abstr.* **9**, 835 (1983).
8. Hishinuma, A., Hockfield, S., McKay, R. & Hildebrand, J.G. *Soc. Neurosci. Abstr.* **11**, 919 (1985).

Jonathan Bacon is in the Genetics and Development Group in the School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK.

Fundamental forces

Are there more than four?

from Alvaro De Rújula

It is usually thought that there are four fundamental forces: the strong 'chromodynamic' force, electromagnetism, the weak interactions and gravitation. The list could be shortened, by invoking known or hypothetical unifications, or lengthened, by recalling that we know quite a bit about the interaction allegedly responsible for the non-vanishing values of particle masses. The conventional number has recently been challenged by claims for the existence of a new fifth force. Geophysical measurements^{1,2} of gravity disagree with laboratory experiments at shorter distances, much as though an intermediate-range force was competing with the gravitational force.

The results of the classical experiment in 1922 of Eötvös and collaborators³ on the equality of gravitational and inertial masses show minute but systematic deviations, correlated with the baryon (proton + neutron) number of the measured substances⁴.

On closer inspection, the geophysical indications turn out to be marginal^{5,6}, and an intermediate-range force interpretation⁴ of Eötvös' results becomes hard to defend^{6,8}. But Eötvös' apparent baryon number effect remains very significant. What could its origin be?

A hypothetical deviation from Newton's law on the attraction between two bodies can be described by an additive

fifth potential, V_5 , of range λ and relative strength α :

$$V(r) = V_N + V_5(\alpha, \lambda) = -G(m_1 m_2 / r) [1 + \alpha e^{-r/\lambda}]$$

where G is the gravitational constant, and V_N is Newton's gravitational potential energy at a distance r . The new force need not couple to mass and α may depend on the nature of the point-like bodies of mass m_1 and m_2 . Yet α is generally assumed constant in the presentation of results that are not explicit comparisons of different materials. The experimental limits on attractive forces ($\alpha > 0$) are summarized⁹ in Fig. 1; the limits on $|\alpha|$ for $\alpha < 0$ are practically the same.

The recent revival of the fifth-force story began with the contention that very close to the 'geological' limit of Fig. 1, although for $\alpha < 0$, there is a set of values of α and λ actually describing a new observed repulsive force. A geological test of Newton's law is obtained by measuring gravity as a function of depth in a mine. The results can be fitted within errors with a value of G that appears to be bigger in the geophysical environment than in laboratory experiments at smaller values of r at which, if $\lambda \gg r$, the extra repulsive force is not exponentially damped: $G_{\text{LAB}} \approx G(1 + \alpha)$. Data¹ gathered in mines at different locations show that the effect cannot be explained by a local gravitational anomaly, that is, a large

density inhomogeneity deep under a particular mine. Moreover, dedicated measurements⁷ in a well-surveyed Australian mine have fitting errors sufficiently small to place the apparent central value of G some 30 (!) standard deviations away from the laboratory measurement. Alas, a recent reanalysis⁵ of the mine data shows that the systematic errors implied by the limited knowledge of the local density distribution are fairly large. From the best-studied mine the geophysical evidence for a fifth force with an 'intermediate' range ($1 \text{ m} \leq \lambda \leq 1 \text{ km}$) is not more significant than two systematic error bars^{5,6}. On this front, there is no longer a serious threat to Newton's peaceful rest.

The fifth force became world news when Fischbach and collaborators⁴ brought attention to an effect, unnoticed for more than 50 years, in the results of the justifiably famous work of Eötvös, Pekar and Fekete³. The operating principle of their device is explained in Fig. 2. The experiment measures torques, but its results, if interpreted as deviations from the equivalence principle, can be re-expressed as normalized differences in the accelerations with which different materials would fall, if set free: $\Delta\gamma_{12} \approx (g_1 - g_2)/g_N$. All the measured $\Delta\gamma$ for different pairs of materials are $< 10^{-8}$, and compatible with zero within a few inconclusive standard deviations. But if the results on $\Delta\gamma$ are plotted against baryon number differences (an unknown concept in Eötvös' time) a linear correlation clearly appears^{3,9}, with a slope that differs from zero by not less than eight overwhelming standard deviations. Misled by Eötvös' presentation in terms of accelerations,

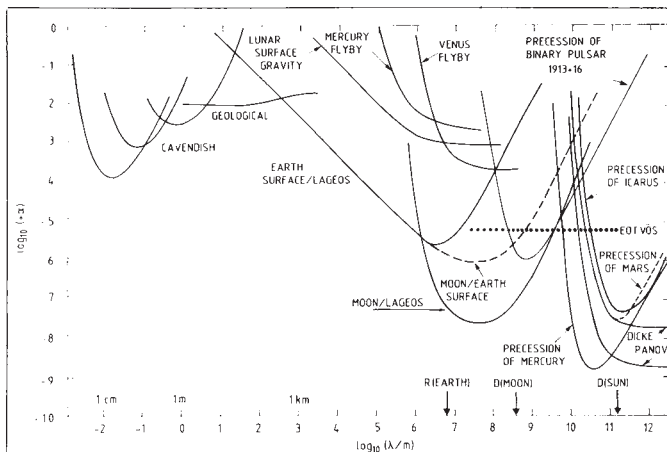


Fig. 1 Limits⁹ on α , the strength relative to gravity of new interactions of range λ . For a given λ the values of α above the lowest continuous curve are excluded. Cavendish, laboratory experiments; geological, measurements down a mineshaft. Three curves stem from comparisons of the gravitational pull of the Earth on a body at its surface, on the Moon and on the artificial satellite Lageos. Curves labelled 'Precession of' reflect the agreement of general relativistic post-newtonian theory and observation concerning the apsidal displacement of the orbits of the binary pulsar PSR1913+16, and of various bodies in our Solar System. Dicke and Panov, comparisons of the pull exerted by the Sun on different materials on the Earth drawn as limits on the particular case of a force coupled to baryon number, rather than mass.

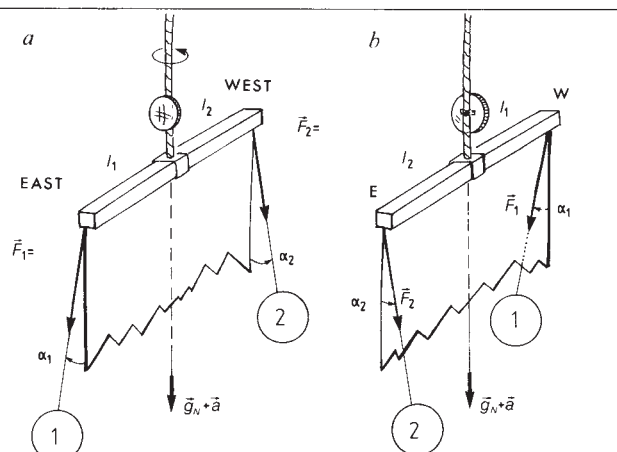


Fig. 2 Eötvös' torsion balance (*a*, twist in suspension $\theta = 0$; *b*, $\theta = \pi$), able to detect deviations from the equivalence principle, the principle of proportionality of inertial and gravitational masses: $m_i/m_f = m_i/m_f$. Acceleration of gravity, $\vec{g}_N$; $\vec{a}$, axifugal acceleration caused by the Earth's rotation; $\vec{F}_j = m_j^g \vec{g}_N + m_j^i \vec{a}$, $j=1,2$, are the balls' weights. The ratio l_1/l_2 is adjusted so that the vertical weight projections are precisely balanced. If the equivalence principle fails, $\vec{F}_1$ and $\vec{F}_2$ are not parallel, and there is a net torque on the hanging wire, detectable as a differential torque ($\propto \cos\theta$) as the whole apparatus is bodily rotated from an east-west to a west-east position.

Fischbach *et al.* proceeded to interpret their gorgeous bibliographical discovery as a fifth force, coupled to baryon number, and exerted by the strata underlying Eötvös' laboratory. In idealized conditions (flat terrain and strata), and for a force with an intermediate range much smaller than the radius of the Earth, this interpretation is incorrect^{6,7}. Indeed, if the equivalence principle is largely correct, all weights in Fig. 2 are parallel and directed along the truly vertical direction: $\vec{g}_N + \vec{a}$. The surface of a quiet sea and, on the average, the superficial land strata, are in isostatic equilibrium orthogonal to $\vec{g}_N + \vec{a}$. Barring local inhomogeneities, a force whose source is this nearby underlying matter is directed in the same direction ($\vec{g}_N + \vec{a}$) as the torsion wire of the balance: it cannot produce a torque around it, QED. A relatively short-range force, exerted by the inhomogeneous surroundings of the experiment, may be responsible for the observed effects, a possibility that is hard to pin down. Although malicious rumour has it that Eötvös himself weighed more than 300 pounds, unspecific hypotheses are not, *a priori*, particularly appealing.

Toying with logic and vectors, we have not really understood Eötvös' non-null results. It is conceivable that some uninteresting variable is disguising itself as baryon number, B . There is a rough correlation, R.H. Dicke points out (Chu, S.Y. and Dicke R.H. *Phys. Rev. Lett.* **57**, 1823; 1986), between B and inverse density, ρ^{-1} , for several of the substances tested by Eötvös. Temperature gradients in the apparatus could perhaps result, via air currents within its enclosure, in a correlation with ρ^{-1} . (To avoid this and other systematic effects, Eötvös flipped the positions of all pairs of materials, and averaged the results.) A second and more 'fundamental' explanation⁸ can be found in terms of a force with a long range much greater than the radius of the Earth. Such a force originates, like gravity, from the Earth as a whole, and it is directed along $\vec{g}_N$. Its effects are equivalent to a material-dependent modification of gravitational mass: Eötvös' balance is sensitive to them. The value of α required by this explanation is plotted in Fig. 1 as the dotted line. By what is presumably a monumental coincidence, there is one point on this dotted line, at $\lambda \approx 4 \times 10^{10}$ metres, that falls in a narrow window not disallowed by astronomical observations. This literally far-fetched interpretation⁹ of

Eötvös' results would correspond to an attractive force, approximately coupled to proton + neutron (or neutron + electron) number, and mediated by the exchange of a new scalar or tensor particle of mass some 22 (!) orders of magnitude lighter than the electron. Finally, it may well be that the many experiments now in progress do not reproduce Eötvös' results, and

we must turn for good this page of the fifth force saga. In that case, Eötvös and collaborators would have carried their secret to their graves: how to gather ponderous evidence for something like baryon number decades before the neutron was found. □

Alvaro De Rújula is in the Theory Division at CERN, 1211 Geneva 23, Switzerland.

Astronomy

Detecting gravitational waves

from David Blair

WHEREAS electromagnetic waves are seen everywhere, gravitational radiation has not been detected. Huge laser-interferometer experiments are being planned to detect gravitational waves (see *Nature News and Views* **321**, 378; 1986), but the technology of cryogenic resonant bar gravitational-wave antennas, the first form of detector to be proposed, has at last reached the stage where long-term coincident operation of several widely spaced sensitive antennas has been achieved. Preliminary, but null, results for the first three-way coincidence experiment have recently been reported*.

In 1960 Joseph Weber (*Phys. Rev.* **117**, 306) outlined the basic design for a resonant bar gravitational radiation antenna. Since the early 1970s a world-wide network of cryogenic resonant bars has been under development. All, following Weber's original design, use highly vibration-isolated metal bars, but now use a low acoustic-loss aluminium alloy or niobium, and are cooled to liquid helium temperatures. In principle a resonant bar antenna is simple: a gravitational wave induces a small phase and amplitude change in the thermal vibration of the antenna. This can be measured above the thermal noise if the quality, or Q , factor is high enough, and if the vibration sensor is sufficiently sensitive. (Q measures the acoustic loss of the antenna. High Q means low damping, and consequently near-perfect, low-noise oscillation.)

The three operating antennas, at CERN near Geneva (operated by the University of Rome), at Stanford University and at Louisiana State University, have a sensitivity to the Riemann curvature or strain amplitude of between 8×10^{-19} and 3×10^{-18} . This sensitivity is sufficient to see most predicted transient gravitational radiation events in the Milky Way galaxy (such as supernovae and the coalescence of binary black holes or neutron stars), but is insufficient to see more distant events in the Virgo cluster and beyond. Because events in our Galaxy are likely to be rare, it was not surprising that

no events have been observed. But the results do demonstrate the power of multiple coincidence analysis to reject seismic noise (W.O. Hamilton, Louisiana State University). Although the individual antenna 'event rate' was 60–70 per day (measured by a predetermined threshold crossing), no triple coincidences were observed, and the nearest two-antenna coincidence had a 0.9-second time difference. As gravitational radiation should travel at the velocity of light, this coincidence must be attributed to chance. However Hamilton emphasizes that the results are "extremely preliminary". With two more antennas expected to begin soon (at the University of Western Australia and the University of Maryland) there is hope that nearly continuous monitoring in the frequency range 700–1,000 hertz will soon become possible. With information from timing and antenna orientation, reasonable directional information should be attainable once signals are detected.

Why are such promises taking so long to be realized? The answer is that nobody, not even the experimentalists themselves, appreciated the difficulties. A whole range of new technology has had to be developed, including a new generation of low-noise direct current superconducting quantum interference devices. These devices are now used as the amplifiers for the superconducting vibration sensors on the antennas that are now operating. Meanwhile, groups in Moscow, Perth and Tokyo have developed parametric transducers, which use superconducting microwave techniques to read out the antenna vibrations, and very high Q -factor antennas. The Perth group reported a Q -factor in their niobium antenna of 2.3×10^8 . After a 'knock', this bar will ring for more than one day.

Everyone is working to push the sensitivity towards 10^{-20} and to push up the antenna bandwidths from the present 1–10 to several hundred hertz. This can be achieved if the transducer impedance can be better matched to the antenna. □

David Blair is in the Department of Physics at the University of Western Australia, Nedlands, Western Australia 6009.

1. Stacey, F.D. & Tuck, G.J. *Nature* **292**, 230 (1981).
2. Holding, S.C. & Tuck, G.J. *Nature* **307**, 714 (1984).
3. Eötvös, R.V., Pekar, D. & Fekete, E. *Ann. Phys., Leipzig* **68**, 11 (1922).
4. Fischbach, E. *et al. Phys. Rev. Lett.* **56**, 3 (1986).
5. Holding, S.C., Stacey, F.D. & Tuck, G.J. *Univ. Queensland preprint* (1986).
6. De Rújula, A. CERN preprint TH.4466 (1986).
7. Bizzi, P.G. Florence Univ. preprint DFF86 No.31 (1986).
8. Milgrom, M. Princeton Inst. Adv. Study preprint (1986).
9. De Rújula, A. CERN preprint TH.4515 (1986).

*11th International Conference on General Relativity and Gravitation, Stockholm, 6–12 July 1986.

Virology

A new human herpesvirus

From Robin Weiss and Carel Mulder

In this week's *Science* (234, 596 and 601; 1986), two papers describe a hitherto unknown herpesvirus isolated from six patients with lymphoma or lymphoproliferative disorders. Zaki Salahuddin, in Robert Gallo's laboratory, first observed some odd-looking giant cells in short-term cultures of peripheral blood leukocytes from three patients with different types of lymphoma — an acquired immunodeficiency syndrome (AIDS)-related B-cell tumour, a cutaneous T-cell lymphoma and an immunoblastic lymphoma. These cells expressed viral antigens. The same virus was subsequently isolated from three further patients, two with lymphadenopathy and one with T-cell acute lymphocytic leukaemia.

Morphologically, the virus particles have the typical appearance of herpesviruses, being 200 nm in diameter, with an outer envelope loosely surrounding an icosahedral nucleocapsid. The genome is a large one (more than 110 kilobases of double-stranded DNA) and thus in the range of other herpesviruses (100–220 kb). A 9-kb probe developed from the

new virus did not hybridize with any of the known human herpesviruses (Epstein–Barr virus, cytomegalovirus, herpes simplex viruses and varicella-zoster virus). The new virus is also serologically unrelated to other primate herpesviruses.

Replication of the virus appears to be restricted to freshly cultivated B cells and for this reason the discoverers have named the virus human B-lymphotropic virus (HBLV). One wonders what these or other investigators will call a human B-lymphotropic retrovirus if such a virus comes to light one day. HBLV does not appear to transform or immortalize B cells, and the isolation of the virus from 3 out of 6 patients with T-cell neoplasia or lymphadenopathy should lead to caution about an aetiological role for this B-lymphotropic virus in the lymphoproliferative disorders described. Two of the six patients were also infected with the AIDS virus (human immunodeficiency virus, HIV), one suffering from AIDS itself, but Salahuddin *et al.* promise further sero-epidemiological data indicating that the risk groups for HBLV infection are not

wholly coincident with HIV. They imply that AIDS-related lymphoma may be aetiologically linked with HBLV. Among 220 healthy donors, only 4 were HBLV seropositive, contrasting with the high prevalence of EBV infection world-wide.

HBLV is the first human herpesvirus to be discovered since Epstein, Achong and Barr observed the Epstein–Barr virus in electron micrographs of cultured Burkitt's lymphoma cells. Lymphoproliferative herpesviruses of new world monkeys, of rabbits and of chickens have been described. The present papers mark just the beginning of investigations into the molecular and cell biology, epidemiology and pathogenesis of HBLV. Once again, Gallo and his team have published provocative new data that will keep many investigators busy for some time to come.

We wonder whether there are more herpesviruses lurking in the human population. The agents of Hodgkin's disease and Kaposi's Sarcoma would be plausible candidates. The search for viral agents is given further impetus by the report on page 814 of this issue (*Nature* 323, 814; 1986) of retroviral association with Kawasaki disease. □

Robin Weiss is at the Institute of Cancer Research, London SW3 6JB; Carel Mulder is on a sabbatical visit from the Department of Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01602, USA.

The demise of a living fossil?

ONE of the best-known examples of a living fossil is the tuatara *Sphenodon punctatus*, found in New Zealand (see figure). *Sphenodon* looks like a medium-sized lizard, but its skull and skeleton appear to reveal primitive characters that have been lost by lizards. It is often said to have remained unchanged from its ancestors of over 200 million years ago, but new research^{1–3} suggests that *Sphenodon* is not as archaic as has been assumed.

What is a living fossil? A common view, stemming from Darwin⁴, is that living fossils are lineages that have survived for a long time and have evolved very slowly, mainly because of the absence of biological competition. Schopf⁵, on the other hand, argues that living fossils probably do not exist as most examples either lack a fossil record or there is no evidence that the extinct forms really are the same as their supposed ancestors.

Although there are no known fossil specimens of *Sphenodon*⁶, how similar is it to the fossil sphenodontians? The earliest known sphenodontians occurred in the late Triassic, about 225 million years ago^{1,3}. Some of these animals, varying in body length from 15 to 35 cm, had snouts and multiple rows of teeth all over the bones of the palate, whereas others had fewer rows of teeth and longer snouts. Most of these

early sphenodontians had teeth fused to the bones of the jaw (the acrodont condition, typical of *Sphenodon*) but others⁷ had some teeth set in a bone groove (the pleurodont condition, seen in many living lizards). The teeth indicate various insectivorous and herbivorous diets.

Later sphenodontians of the Jurassic (144–208 million years ago) include long-bodied marine animals⁸ which reached lengths of 1.5 m; tiny terrestrial species less

than 20 cm long; and bizarre Jurassic and early Cretaceous genera^{6,7} which had transversely broad teeth and were probably herbivores. The Sphenodontia of the Mesozoic were diverse, consisting of tiny insectivores, moderate-sized herbivores and several specialized marine forms.

It is clear that *Sphenodon* can no longer be treated as the last remnant of a lineage of living fossils. Several fossil sphenodontians show lizard-like characters that are absent in *Sphenodon*, whereas others developed unique specializations not seen in any other group. The Sphenodontia as a whole cannot be called living fossils by any definition, while *Sphenodon* itself can only be so described in terms of its present-day low diversity and restricted geographical distribution. It does not show primitive characters — Whiteside³ and I⁸ estimated that *Sphenodon* displays almost 20 advanced features when compared with the nearest relatives of the Sphenodontia. It cannot have persisted unchanged for 100–200 million years. Michael J. Benton

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Sphenodon punctatus from Stephens Island, New Zealand (Heather Angel).

1. Fraser, N.C. *Palaeontology* 25, 709 (1982); 27, 575 (1984); 29, 165 (1986).
2. Carroll, R.L. *Paleontographica* A189, 1 (1985).
3. Whiteside, D.I. *Phil. Trans. R. Soc. B* 312, 379 (1986).
4. Darwin, C. *On the Origin of Species* (Murray, London, 1859).
5. Schopf, T.J.M. *A. Rev. Earth planet. Sci.* 12, 245 (1984).
6. Throckmorton, G.S., Hopson, J.A. & Parks, P. *J. Paleontol.* 55, 586 (1981).
7. Rasmussen, T.E. & Callison, J. *Paleontol.* 55, 1109 (1981).
8. Benton, M.J. *Zool. J. Linn. Soc.* 84, 97 (1985).

Even plants excrete

SIR—Excretion is usually omitted from orthodox accounts of plant physiology. It has been argued that the 'inactivity' of plants renders excretion unnecessary¹. But, although it might be argued that the rate of metabolic activity in a non-motile autotroph produces a reduced excretory load, there is no reason to suggest that it should be altogether absent. I postulate that leaf fall (abscission) is the mechanism of excretion in vascular plants.

The traditional view of leaf fall centres on the occlusion of the vascular bundles by a seasonally produced cork layer, so that the leaf dies and is detached from the plant body. However, the shedding of leaves depends on other determinants: thus, abscission may occur in plants where an abscission layer has not formed, and leaf fall may not take place in species where an abscission layer is present². 'Senescence' is widely used as a synonym for yellowing, though it is hard to conceive of a sixth-month leaf as 'senescent' when the (older) plant which produced it is in its prime. The changes that take place in the yellowing leaf are a coordinated sequence of events and although some vital indicators (including protein content and photosynthetic activity) fall during the process, others increase. Among these are levels of soluble carbohydrate and nitrogen (associated with translocation) and, significantly, RNA content and respiration rate³.

Supportive of my view that leaf yellowing has an anabolic component is the observation that cycloheximide (which inhibits protein synthesis) arrests the process⁴. The classical reason given for leaf fall is that it obviates damage to a plant during winter. The evergreen species demonstrate that this is not obligatory, and with deciduous conifers (such as *Larix*) and genera like *Lonicera*, which include both evergreen and deciduous species of outwardly similar appearance, the picture becomes less clear. If evergreen leaves are anatomically adapted to resist winter conditions they need not be shed, but in fact they are subject to leaf fall — although it takes place continually, rather than on a seasonally-mediated basis. The shedding of all leaves at predetermined intervals suggests that functional constraints are fundamental. Non-laminar leafy structures (such as tendrils, which develop from petioles) are often retained, whereas leaf-like organs developed from stem progenitors (including phylloids) are shed, even though they are not leaves.

It appears that these organs have two discrete functions. In addition to acting as the plant's metabolic centre, the leaf is also the structure that — at the end of its useful metabolic life — is systematically stripped of its vital constituents and charged with metabolic waste materials.

The anthocyanins and tannins which are synthesized, like the oxalates which accumulate in yellowing leaves, are in reality among the excretory products the plant needs to discard. The yellowed leaf becomes an 'excretophore', and the shedding of the leaf may be seen as the plant's excretory mechanism.

BRIAN J. FORD

Mill Park House,
57 Westville Road,
Cardiff CF2 5DF, UK

1. Brocklehurst, K.G. & Ward, H. *A New Biology* 114 (Hodder & Stoughton, London, 1977).
2. Meyer, B.S. & Anderson, D.B. *Plant Physiology*, 733 (Van Nostrand, New York 1952).
3. Bidwell, R.G.S. *Plant Physiology*, 485 (Macmillan, London, 1974).
4. Rhodes, M.J.C. in *The Biochemistry of Plants* (ed. Davies, D.D.) 431–437 (Academic, London, 1980).

Human $^{134}\text{Cs}/^{137}\text{Cs}$ levels in Scotland after Chernobyl

SIR—Following the accident at Chernobyl on 26 April 1986, and the subsequent appearance of caesium radioisotopes in the food chain, we have measured the ^{134}Cs and ^{137}Cs whole body activities in eighteen adult subjects, thirteen males and five females, living in the Glasgow area. The measurements were made between 11 June and 18 July 1986 using a highly-sensitive whole-body counter with a ring of six large sodium iodine crystal scintillation detectors surrounding the subject and scanning from head to foot in 1,000 seconds.

In the gamma-ray spectrum for the subject with the highest level of contamination (Fig. 1), the main contaminant photopeaks are at 620 and 800 keV and the photopeak of the naturally occurring radioisotope of potassium, ^{40}K , is at 1,460 keV. ^{134}Cs emits three main gamma rays at 570, 605 and 800 keV whereas ^{137}Cs decays to a short-lived daughter, $^{137\text{m}}\text{Ba}$, which emits a 662 keV gamma ray. It has been assumed that the peak at 800 keV is due solely to ^{134}Cs , but that the peak at around 620 keV represents an unresolved com-

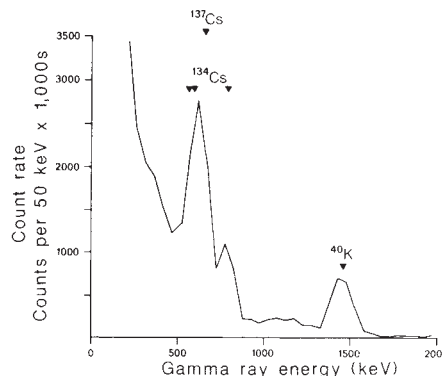


Fig. 1 Gamma-ray spectrum for the subject with the highest levels of ^{134}Cs and ^{137}Cs contamination (^{134}Cs whole body activity = 285 Bq, ^{137}Cs whole body activity = 663 Bq).

bination of the 570 and 605 keV peaks from ^{134}Cs and the 662 keV peak from ^{137}Cs (via $^{137\text{m}}\text{Ba}$). Because the spectra from the three radionuclides overlap, we converted the count rates in Fig. 1 into absolute activities of ^{134}Cs , ^{137}Cs and ^{40}K by observing the spectra obtained (count rate per energy interval per bequerel) with water-filled anthropometric phantoms of differing weights (35–90 kg) filled with known activities of ^{134}Cs , ^{137}Cs and ^{40}K .

The mean ^{134}Cs activity was 172 ± 73 Bq (mean ± 1 s.d.) with no significant difference between males and females. The mean ^{137}Cs activity was 363 ± 172 Bq and the mean value for males (403 ± 172 Bq) was significantly higher than that for females (256 ± 128 Bq) ($P \leq 0.05$). The group mean whole body activity of the naturally occurring ^{40}K was $4,340 \pm 532$ Bq for males and $2,859 \pm 576$ Bq for females. These activities were equivalent to total body potassium levels (TBK) of $3,647 \pm 447$ mmol for males and $2,403 \pm 484$ mmol for females.

The ratio of radioactive Cs to K is conventionally taken as a measure of radioactive Cs contamination independent of body size and sex. By this test, there was no significant difference between the sexes for either radioisotope (total group mean values of 0.052 ± 0.021 Bq mmol⁻¹ for $^{134}\text{Cs}/\text{TBK}$ and 0.109 ± 0.047 Bq mmol⁻¹ for $^{137}\text{Cs}/\text{TBK}$). We do not have pre-Chernobyl ^{134}Cs or ^{137}Cs activities for our subjects, but we obtained a mean $^{137}\text{Cs}/\text{TBK}$ ratio of 0.025 Bq mmol⁻¹ for three males measured in 1983 whereas a ratio of 0.0371 Bq mmol⁻¹ was recorded for a group of 69 subjects resident in mainland Scotland in 1978–79¹.

A significant proportion of the observed contamination is expected to come from the ingestion of milk and dairy produce. We have fitted local ^{137}C milk activities for the period 6 May to 17 July, supplied by the National Radiological Protection Board (Scottish Centre), to a single exponential function of time characterized by an initial activity of 31.2 Bq l⁻¹ and a half life of 60.8 days. Therefore, as the average adult in the Glasgow area consumes approximately 0.33 l milk per day², it is possible to predict the average activity of ^{137}Cs ingested from milk as a function of time and to use this as the input function to a simple model of Cs metabolism³, in which 90% of ingested Cs enters a single pool with a biological half life of 110 days and the remaining 10%, which also enters the body, leaves quickly with a half life of 2 days. On the assumption that contamination occurs only from milk ingestion, the model predicts that ^{137}Cs whole body activity rises to a maximum of 390 Bq after 110 days and declines to half that over the next 200 days; the predicted value at the midpoint of the present measurement period is 318 Bq. If we postulate that the

initial ^{134}Cs to ^{137}Cs ratio was 0.5, then the ^{134}Cs whole body activity rises to a maximum of 178 Bq after 100 days and reduces to half that in the next 180 days, whereas the predicted value at the midpoint of the present measurement period is 152 Bq.

This simple model, based on milk consumption alone, predicts whole body activities of ^{134}Cs and ^{137}Cs at the time of our present measurements with reasonable accuracy. Given seasonal changes in food production and consumption, the longer term validity of the model cannot be guaranteed, but it is instructive to calculate the whole body radiation doses based on the activity-time predictions of the model.

The predicted average additional effective dose equivalent for 1986 due to the contamination from Chernobyl is 4.7 μSv for ^{134}Cs and 6.9 μSv for ^{137}Cs (+ $^{137\text{m}}\text{Ba}$) whereas the predicted average committed effective dose equivalent is 7.2 μSv for ^{134}Cs and 11.6 μSv for ^{137}Cs (+ $^{137\text{m}}\text{Ba}$). These predicted doses are relatively small compared to those resulting from naturally occurring ^{40}K , (174 μSv per annum for males and 116 μSv per annum for females) and the combined committed dose for both Cs radioisotopes amounts to only 1% of the total average annual effective dose equivalent due to natural background radiation⁴.

I thank Mr D. Smith of the NRPB for his help.

W.S. WATSON

Department of Clinical Physics
and BioEngineering,
Southern General Hospital,
Govan Road,
Glasgow G51 4TF, UK

1. Williams, E.D. *et al.* *Health Phys.* **40**, 1-4 (1981).
2. Ministry of Agriculture, Fisheries and Food Household Food Consumption 1984: A. Rep. of the National Food Survey Committee (HMSO, London, 1986).
3. International Commission on Radiological Protection Limits for Intakes of Radionuclides by Workers ICRP Publ. 30 (Pergamon, Oxford, 1979).
4. Fry, F.A. *et al.* *NRPB R121* (HMSO, London, 1981).

Origin of HTLV-I virus in Japan

SIR—If the human T lymphotropic virus I (HTLV-I) was brought to Japan from Africa by the Portuguese in the sixteenth century as in the proposal of Gallo *et al.*^{1,2}, which is the subject of controversy³⁻⁶, the virus should be prevalent in India, Ceylon and Malacca, where the Portuguese constructed their bases long before arriving in Japan. Yet, recently, we tested 90 sera in Bombay for HTLV-I antibody and found none of them to be positive⁷.

Portuguese missionaries began their activities in Japan in 1559 and the Portuguese dominated trading in Japan until the seventeenth century. Japanese history has no record of African descendants in Kyusyu. I therefore agree with the hypothesis of Ishida and Hinuma^{6,8} that

HTLV-I was originally carried by prehistoric Japanese (Asiatic, Caucasoid, Ainu and Ryukyuan). Interestingly, the Ainu are ethnically related to the Eskimos, who have a high prevalence of HTLV-I, according to Gallo and his colleagues⁹.

So, are all HTLV-I positive populations in Japan directly related to Ainu or Ryukyuan? I suggest that HTLV-I was once enclosed in Okinawa and was first transmitted to Kyusyu through the trade between Okinawa and southern province of Kyusyu (Kagoshima). Further expansion of HTLV-I to other coastal areas of Japan might have been carried out by fishermen along the ocean current *Kuroshio*. Until 100 years ago, the movement of ordinary people beyond province borders was strictly limited by *Shogun* but, even then, fishermen could travel rather freely by ship. The situation is well demonstrated in Kochi prefecture¹⁰ where a higher rate of HTLV-I positivity is found in the east and west fishermen's bases than in the central area. Thus, it is clear that evidence for the hypothesis of an African origin of HTLV-I in Japan is still lacking.

HIROKUNI TAGUCHI

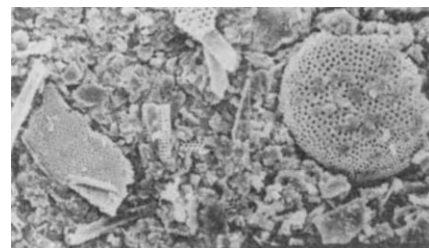
Department of Internal Medicine,
Kochi Medical School,
Kochi 781-51, Japan

1. Gallo, R.C., Sliski, A.H. & Wong-Staal, F. *Lancet* **ii**, 962-963 (1984).
2. Wong-Staal, F. & Gallo, R.C. *Nature* **317**, 395-403 (1985).
3. Rosenior, J. *Nature* **318**, 728 (1986).
4. Gallo, R.C. & Sliski, A.H. *Nature* **320**, 219 (1986).
5. Kantha, S.S. *Nature* **321**, 733 (1986).
6. Ishida, T. & Hinuma, Y. *Nature* **327**, 504 (1986).
7. S.H. Advani *et al.* *Ind. J. med. Res.* (in the press).
8. Ishida, T. *et al. J. Infect.* **11**, 153-157 (1985).
9. Robert-Guroff, M. *Int. J. Cancer* **36**, 651-655 (1985).
10. Taguchi, H. *et al. Lancet* **ii**, 1029 (1983).

Diatom mystery

SIR—The mystery object in Wolstenholme's chromosome preparation¹ is a fragment of the silica skeleton of an alga known as a diatom. We had significant problems with contamination from this tiny organism's remains for several years. Eventually we accidentally discovered that the source of the contamination was diatomaceous particles from rubber bulbs placed on Pasteur pipettes, used for the harvesting phase of chromosome preparations². Diatomaceous earth is apparently used by some manufacturers in the production of rubber goods.

Diatom particles clung to many cells, cutting their membranes and releasing the contents (in the same manner that diatomaceous earth is used as an organic insecticide). This diminished the numbers of well-spread chromosome metaphases. The particularly vulnerable cells in this procedure were those in metaphase (and therefore without a nuclear membrane) which were under internal osmotic pressure due to prior hypotonic treatment. The greater the dose of diatomaceous frag-



Diatom fragments on inner surface of rubber bulb. Left, $\times 880$; right, $\times 1,190$.

ments, the more cells in metaphase that were affected, and fewer spreads were analysable.

THOMAS S. MCCONNELL

GERALD T. ALLGOOD

PATRICIA O. BACA

GLENDIA E. MILLER

ROBERT E. WATERMAN

Departments of Pathology and Anatomy,
The University of New Mexico
School of Medicine,
Albuquerque, New Mexico 87131, USA

1. Wolstenholme, J.W. *et al. Nature* **323**, 300 (1986).
2. McConnell, T.S. *et al. Karyogram* **11**, 59-60 (1985).

SIR—When I showed the crossword puzzle-like but only 10-micrometre "mystery object amid the chromosomes" reported by Wolstenholme *et al.*¹ to my mythical friend Dr Orpheus of the Institute of Neuropoetics, he responded with the following:

The late Al Lehninger, of textbook² fame,
In 1966 was deemed insipid
By players of nucleic acid's game
For speculating³ that a membrane's lipid
Might code environmental information
Across its two-dimensional array,
A thought that called forth righteous
indignation
From priests of one-dimensional DNA,

But just as viruses, which travel light,
Have usefully evolved the economic
Reading frame-shifts, to the left or right,
And then both left and right, or palindromic,
So that, as if possessed of frugal wits
That make the most of being just a line
Of four-state points that can't bear many bits,
The Palimpsest-like viruses do fine,

So, likewise to save space, eukaryotic
Cells, to which the neurones dictate chatter,
Must complement their chromosomes' demotic
Morse-like coding of genetic matter
By interlocking news Across and Down
Their protein-punctured lipid membranes,
As shown by Wolstenholme *et al.*¹ . . .

Don't frown,
Oh 1-D dogmatists! (Did God give *them*
brains?)

TED MELNECHUK

Helicon Foundation,
4622 Sante Fe Street, San Diego,
California 92109, USA

1. Wolstenholme, J. *et al. Nature* **323**, 300 (1986).
2. Lehninger, A.L. *Biochemistry*, 2nd edn (Worth, New York, 1975).
3. Lehninger, A.L. in *Neurosciences Research Symposium Summaries* Vol. 1 (eds Schmitt, F.O. & Melnechuk, T.) 55 (MIT Press, Cambridge, 1966).

Goodall and Gombe

W.C. McGrew

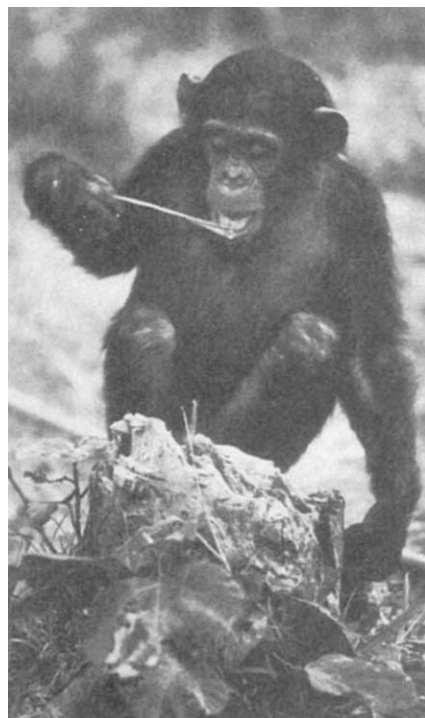
The Chimpanzees of Gombe: Patterns of Behavior. By Jane Goodall.
Harvard University Press: 1986. Pp. 673. \$30, £19.95.

JANE Goodall is simply the most famous primatologist ever. Even if someone has never read her *Animal Behaviour Monograph* (1968), reporting her scientific studies of chimpanzees, or her more popular books, *My Friends the Wild Chimpanzees* (1967) and *In the Shadow of Man* (1971), she or he has probably seen one of Dr Goodall's wildlife documentary films. This alone means that any book of hers would deserve attention, for in a certain sense she defines the science for the world at large, in much the same way that Margaret Mead did for cultural anthropology.

In this case, the book will generate even more excitement, for it is the long-awaited updating of the Gombe story. The author produced her three previous books in little over ten years after starting in 1960 her pioneering field study of the long-haired *Pan troglodytes* of western Tanzania. But in the past 15 years there has been nothing, at least in book form. So, to which of her earlier books is this a sequel? Perhaps surprisingly, it tries to be all three. Like *My Friends* it is lavishly illustrated, with over 350 photographs. Like the monograph, it is full of tables, graphs and references. Like *Shadow*, it is a rich description of the lives and times of a community of individuals. The ambitiousness of the attempt is the source of both its strengths and weaknesses.

There are 20 chapters in the book, plus five appendices. Thirteen of the 20 are "data chapters", in that they report the analyses of quantitative data. The topics covered are what one would hope to find: demography, dominance, hunting and sex, territoriality, object manipulation, and so on. These bring up to date the findings of recent years, and in some cases drive home a point which Dr Goodall has stressed repeatedly: that had study of Gombe's apes stopped after ten years (which is after all already a very long study by usual standards), our conclusions would have been incomplete and even misleading. The most graphic example is that of the extermination of the Kahama community by the Kasakela community, in a ruthless, one-by-one fashion. There was no hint of this until 1974, and till then systematic pongicide was undreamed of. Other results serve to confirm earlier suspicions, for example that female chimpanzees may also hunt, kill and eat mammalian prey, even fast-moving arboreal monkeys, under certain circumstances. Sex differences in predation persist, but simplistic

generalizations (by this reviewer as well as others) about which sex does what are shown to be just that. Finally, other results are remarkable by their consistency: little has emerged about diet or the use of tools which was not known in the first 10–15 years. Instead, what is impressive is the conservatism of the material culture of



Tool use — Flint, son of Flo, fishing for termites with a blade of grass. (H. van Lawick; reproduced from *The Chimpanzees of Gombe*.)

wild chimpanzees, in that no innovation has been seen in 25 years.

The wealth of data is sometimes almost staggering: averages of up to 638 hours of observation *per subject per year*; 59 individuals followed from birth; 374 kills of vertebrate prey. Sadly, however, the analyses do not do justice to the data-set. Much hard-won information remains unreported, unanalysed and perhaps even untabulated because of Dr Goodall's resistance to using a computer. When data are presented, they are often difficult to interpret, as means (but not variances) and frequencies (but not rates) are often all that is given. Data are usually pooled across subjects, as for example when looking at the age and sex differences, so that independence of observations is compromised. Statistical tests are few — the first does not appear until p. 259 — and

largely unexplained and mostly wrongly applied. Arguably, this might not matter in a semi-popular account, but two main points persist: one is that an immense amount of work has gone into data-crunching, and it is a pity to see this ill-served. More seriously, it is usually not possible to tell whether apparently key results are real or not. For example, the graphs comparing male and female feeding times are said to be similar; in fact they are statistically significantly different. Or, it is said that distances travelled are much shorter on the day after a long excursion, but the tabulated data show such sequences to be random. One wonders if the book was ever scientifically edited.

The non-quantitative chapters are of mixed quality. The one on laboratory studies of chimpanzees skims selectively over well-known ground, focusing mostly on older work. Early "pongo-linguistics" is accepted uncritically, but (for example) Savage-Rumbaugh's elegant recent work on symbolic abilities is virtually ignored. The chapter might better have been omitted altogether, as it is of doubtful relevance. On the other hand, the chapter on "who's who" is masterly. In 18 pages, Dr Goodall presents pen-portraits of 29 chimpanzees with imagery that serves to keep the characters vividly alive throughout the book. A keen eye for individual differences has always been one of the author's strengths, and this is nowhere better shown than in the chapter on social awareness. She uses the apt anecdote at its sharpest to tackle convincingly the trickiest questions of social manipulation such as intentional deception.

All in all, the result is monumental, in several senses of the word. First, the book is massive: almost 700 pages, in large format with a smallish typeface, so that the text alone exceeds 350,000 words. Despite this it is priced so reasonably that even undergraduates will be able to afford it. Secondly, it chronicles 25 years of continuous research on our closest living relations. It is both comprehensive and comprehensible (she writes as clearly as ever), and it is the best such effort since Schaller's *The Mountain Gorilla*. Finally, it is a celebration of a cast of unforgettable characters, some now dead like old Flo, but others now matured, like Goblin, an infant when the study began but now the dominant male. Few people (Stella Brewer and Frans de Waal are two others who come to mind) can really enter the mind of the chimpanzee and then emerge to write about it well, but Jane Goodall is indisputably the best. □

W.C. McGrew, a Senior Lecturer in the Department of Psychology, University of Stirling, Stirling FK9 4LA, UK, is currently a visiting faculty member in the Departments of Anthropology and Biology, University of New Mexico, Albuquerque, USA.

Snaring quarks

J.H. Mulvey

The Particle Hunters. By Yuval Ne'eman and Yoram Kirsh. *Cambridge University Press*: 1986. Pp. 272. Hbk £25, \$49.50; pbk £7.95, \$13.95.

As a concept the atom is more than 2,000 years old, but it is less than 200 years since Dalton's quantitative studies of chemical reactions gave it an observational basis. The original "particle hunter" was J.J. Thomson who discovered the first sub-atomic particle, the electron, in 1897. Soon after, in the years between 1910 and 1932, the nature of the atomic nucleus was revealed largely by the work of Rutherford and his collaborators. Together with the accompanying development of quantum mechanics and special relativity, which revolutionized our understanding of the laws of physics, these discoveries underlie much of modern technology and our growing power to manipulate the molecules of life.

In their book, Ne'eman and Kirsh introduce the general reader to the micro-world of particles. They start with Democritus and, passing by way of the gold-leaf electroscope, the conundrum of particle-wave duality, the enigma of a left-right asymmetry in nature, and the invention of particle accelerators, which allowed physicists to become more like farmers than hunters, they arrive at the explosion of particle species found, mainly, in the late 1950s and early 1960s. At the time, these observations seemed to confound all hope of rational explanation. Then, in 1964, Ne'eman and, independently, Gell Mann brought confusion to an end with their classification scheme based on the group SU(3), so performing for particle physics what Mendeleev had accomplished for atoms. This in turn soon led to the concept of quarks as constituents of all particles such as the proton and neutron.

The story is well told, apart from a few weak patches, and is enlivened by a sprinkling of personal reminiscences. Although their approach is non-mathematical, the authors do not condescend by evading some of the harder concepts.

The remaining 40 or so pages are not enough to do justice to all the triumphs which have followed, and the pace becomes quite breathless when telling of the recent discoveries of the W and Z bosons at CERN. But this book is a very valuable addition to the growing library bringing to the non-professional reader an appreciation of the ideas and methods used in the search for an understanding of the structure of matter. □

J.H. Mulvey is a Senior Research Officer in the Nuclear Physics Laboratory, University of Oxford, Keble Road, Oxford OX1 3RH, UK.

Gödel: incomplete success

David Miller

Collected Works of Kurt Gödel. Volume I, Publications 1929–1936. Edited by Solomon Feferman *et al.* *Oxford University Press*: 1986. Pp. 474. £25, \$35.

IN HIS doctoral dissertation of 1929, Kurt Gödel established the completeness of the usual formulations of elementary logic. In the following year he proved the incompleteness of every effective formulation of number theory. These two results, the second as devastating and unexpected as the first was satisfying, were announced to the mathematical public by the 24-year-old Gödel on successive days at the second Conference on Epistemology of the Exact Sciences (*Tagung für Erkenntnislehre der exakten Wissenschaften*) held in Königsberg in September 1930. There can be no doubt as to their cardinal importance in the history of modern logic.

The gist of Gödel's incompleteness theorem is this: there is no effective way of axiomatizing the set of true statements about the natural numbers, either in first-order logic (which, by Gödel's completeness theorem, suffices for all correct deductions at that level) or in higher-order logic (type theory). That is, for any (consistent) axiomatic system A, including any extension of Peano arithmetic (PA), there are infinitely many arithmetical truths not among its theorems. Gödel showed that one such truth is a statement G that, within PA (or whatever axiomatic system A is under consideration), is provably equivalent to the assertion that "G is not a theorem of A". His demonstration relies on showing that, provided that the system

of axioms is effectively given, the correctness of any arithmetical proof can be checked by arithmetical methods. For then if G is a theorem of A there is a proof G of it, so that we can prove in A that "G is a proof of G", thus proving in A that "G is a theorem of A"; which is to say that we can prove not-G in A. Thus if G is a theorem of A so also is not-G. So if A is consistent, G is not a theorem of A. But this is what G says, so G is true.

Gödel's second incompleteness theorem (discovered also by von Neumann a few weeks after Königsberg) is obtained by showing that the above result — "if PA is consistent, G is not a theorem of PA" — is itself a theorem of PA. For then if "PA is consistent" is a theorem of PA, so also is "G is not a theorem of PA"; that is, G is, which it is not. So "PA is consistent" is not a theorem of PA. Like the first incompleteness theorem this result applies to all effective extensions of PA. It rocked, but did not quite capsize, the hopes of Hilbert's school that it would be possible to show by proofs referring only to finite objects the consistency of the bulk of classical mathematics.

Gödel's published works will be issued as two volumes of his *Collected Works*, to be followed eventually by an unstated number of volumes containing unpublished manuscripts, lecture notes, and selections from his notebooks and correspondence. All items originally written in German will be reproduced with a translation on the facing page. This first volume contains Gödel's dissertation, and also a published version of it, slightly but tellingly different; his great paper on incompleteness; lecture notes on incompleteness (taken by Kleene and Rosser at Princeton in 1934); the text of his remarks at Königsberg; a brief note on what are today called speed-up theorems; two papers on the decision problem for elementary logic;

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Bird's eye view of cygnets of the mute swan, Cygnus olor. The picture is reproduced from The Mute Swan, by Mike Birkhead and Christopher Perrins, an account of both the biology of the species and the laws and customs associated with swan ownership. Publisher of the book is Christopher Helm, London, price is £13.95.

twelve other papers, mostly inspired by discussions at Menger's colloquium, on intuitionistic logic and arithmetic, other aspects of propositional logic, projective and differential geometry, and even economics; abstracts; and 33 reviews. Gödel wrote with marvellous clarity and conciseness, and everything here is a delight to read.

This volume also contains a 36-page essay by Feferman on Gödel's career, and introductory notes on all the main writings and some of the lesser ones. Most of this editorial material is enlightening and helpful, but despite the extraordinary care that has obviously been lavished on the preparation of the book, there are a handful of unfortunate (though minor) lapses.

For example, on p.19 Feferman intimates that the general result stated (but not proved) by Gödel in a 1936 paper on the lengths of proofs is indeed a result, whilst Parikh's note (p.395) says that the question is open. In the note by Dreben and van Heijenoort on the completeness theorem for elementary logic, Post's conjecture that "a complete symbolic logic is impossible" is described as an anticipation of the Gödel incompleteness theorem.

This remark is likely to puzzle anyone who does not appreciate that the incompleteness of elementary number theory implies the incompleteness of higher-order logic, something that the authors of the note fail to mention. Again, Kleene's first muster-roll (p.138, note k), unlike his second (p.343, note e) of the postulates of elementary PA, neglects to include the recursion equations for sum and product. There are also a number of points at which the notes devote more attention to explaining things that Gödel himself explains clearly than to elucidation of issues that to most readers will be much more mysterious.

Except for Gödel's dissertation and the papers on the decision problem, the principal works reprinted here have all appeared before in English translation, in Jean van Heijenoort's sourcebook *From Frege to Gödel* (Harvard University Press, 1967) or in Martin Davis's *The Undecidable* (Raven, 1965) or both. But this book is unique, and every philosopher or historian of logic will be determined to possess it. □

David Miller is Senior Lecturer in the Department of Philosophy, University of Warwick, Coventry CV4 7AL, UK.

Biological controls

Tim Harris

Biotechnology: An Industry Comes of Age. By Steve Olson. *National Academy Press/Wiley*:1986. Pp.120. Pbk \$9.95, £5.30.

It is difficult to believe that it's now 11 years since the Asilomar conference, held in Pacific Grove, California, to discuss the potential ramifications of the then-new techniques of genetic manipulation. A good deal has happened in the intervening period. The essence of the biotechnology industry which has grown up from the seminal discoveries of recombinant DNA and monoclonal antibodies has been captured in this little book. It consists of ten short chapters, reviewing the proceedings of another conference (like biotechnology itself, conferences on the subject continue to proliferate) held to mark the tenth anniversary of Asilomar.

Olson is a professional science writer and, as might be expected, the prose flows well and is refreshingly free of "biohype", although I found the journalistic style of including direct quotations from speakers irritating at times. Most of the aspects of this fledgling industry are covered, from the technologies themselves and what they can do, to their impact on human health care and agriculture, through to patent and regulatory aspects including those to do with human gene therapy. Oddly, the longest chapter is concerned with these regulatory issues — particularly

the role of the Food and Drug Administration and the Environmental Protection Agency. The American bias which pervades the book is particularly evident here and also in the chapter comparing biotechnology in the United States with that in Japan.

Depending on one's interest, it might be argued that the book is slightly unbalanced because the technological aspects are much more superficially treated than governmental issues. This is either a reflection of the legitimate interests of the author or, more likely, the nature of the conference from which the book was derived. The brevity of the coverage of some technological points might leave the non-biotechnologist with more questions than answers, but the provision of some references at the end of each chapter should provide additional, although not extensive, trails to follow. The middle of the book is filled with some 20 colour plates of high quality, including an amusing one purporting to show flasks containing bacteria producing interferon. To my eye there was more broth than bugs, and certainly no bubbles: otherwise the book seems quite error free.

For people interested more in the regulatory and ethical issues surrounding biotechnology, this book is a good place to start. But those looking for explanation of the scientific aspects should not expect as much technical detail as the title might promise. □

Tim Harris is Head of the Molecular Biology Section at Celltech Ltd, 250 Bath Road, Slough SL1 4DY, UK.

GENE RESEARCH

THE GENE Its Structure, Function, and Evolution

by Lawrence S. Dillon

Dillon presents a unified analysis of the gene and its surrounding sequences. He examines the gene and its functions from a broadly comparative basis, with investigations of prokaryotes, eukaryotes, viruses, organelles, protists, and animals.
0-306-42319-7/885 pp. + index
ill./1987/\$95.00/\$114.00 outside US & Canada
text adoption price on orders of six or more copies: \$49.50

GENE TRANSFER

edited by Raju Kucheralapati

An in-depth look at genetic transfer into mammalian cells through parasexual systems, such as somatic cell hybridization, microcell fusion, microinjection into embryos, and homologous and intrachromosomal recombination.
0-306-42325-1/441 pp. + index
ill./1986/\$59.50/\$71.40 outside US & Canada
text adoption price on orders of six or more copies: \$35.00

GENETICS, DEVELOPMENT, AND EVOLUTION

edited by J. Perry Gustafson, G. Ledyard Stebbins, and Francisco J. Ayala

This new volume explores the question of how the genetic instructions encoded in DNA become expressed in the morphological, physiological, and behavioral features of multicellular organisms through an ordered sequence of events that extends from the initial cell division of the zygote. A volume in the Stadler Genetics Symposium Series.
0-306-42268-9/proceedings/374 pp.
ill./1986/\$49.50/\$59.40 outside US & Canada

Plenum Publishing Corporation
233 Spring Street, New York, N.Y.
10013-1578

In the United Kingdom:
88/90 Middlesex Street
London E1 7EZ, England

THE LANGUAGE OF SCIENCE
Plenum
PUBLISHING CORPORATION

Reader Service No.4

Great expectancies of emotion

Eric A. Salzen

Learning and Emotion: A Biological Synthesis. Volume 1, Evolutionary Processes. By Peter J. Livesey. *Lawrence Erlbaum:1986. Pp.312. \$32.50, £22.*

EMOTION has been variously seen as providing emergency support responses, energizing and response amplification, motivation through incentive and reinforcement, and adaptive instinctive responses, or as being non-functional response disorganization. In this book, Livesey sets out to establish emotion as a system for registering what is or is not relevant for learning. To do this he reviews the evolution of the brain, learning and emotion, with emphasis on species putatively related to the evolutionary line leading to man.

Part I gives an elementary introduction to evolution and a thumbnail sketch of the evolution of man drawn from literature of the 1950s and 1960s. Part II, the longest, describes the evolution of the nervous system from amoeba to man, based on literature of the 1960s and 1970s. It gives a useful summary for the mammalian brain. Part III critically reviews comparative studies of learning in vertebrates, and covers much the same ground as E.M. Macphail's *Brain and Intelligence in Vertebrates* (Clarendon, 1982) and *Animal Intelligence*, edited by L. Weiskrantz (Clarendon, 1985). Livesey, however, is more selective and considers the learning-task performance studies of traditional comparative psychology, Bitterman's work on the systematic variation of a single learning-task, and the theoretical views of Thorpe, Lorenz and Razran on the evolution of learning. Unlike Macphail, Livesey concludes that there has been a qualitative change in learning processes and that "expectancy" is a crucial concept in this respect.

In the fourth and final part, Livesey gives his views on the contribution of affect to this evolution of learning. He begins by defining emotion as "a reactive state evolving from the confluence of the affects of reward and punishment and of expectancies generated through associative learning" (p. 231). After a brief and selective consideration of some literature on emotion, Livesey presents his scheme of the evolution of affect. He claims that: (1) the brain reward/punishment system postulated by Olds does indeed promote feelings or signals of the effectiveness of behaviour and is present in all vertebrates; (2) the evolution of learning involving the anticipation of outcomes and the matching of expectancies and outcomes pro-

vides a new feeling system that coincides with the evolution of mammals; and (3) these feelings can in turn modify expectancies and be externalized as social signals of emotional states.

Livesey thus effectively matches his earlier definition of emotion, no doubt to his satisfaction and pleasure. His thesis implies that the evolution of emotion depended on the evolution of learned expectancies. He also proposes that the function of emotion is to feed back and influence expectancies and so act as a "teaching mechanism". If emotion guides learning as Livesey implies, one wonders what guided Oscar Wilde when, in *The Picture of Dorian Gray*, he wrote: "The advantage of the emotions is that they lead us astray". Livesey's theory fails to account for the evolution of emotional behaviours which are present throughout the vertebrates and are especially evident in birds,

whose evolutionary origin and brain structure are quite different from those of mammals, to whom, apparently, emotion *sensu strictu* is restricted.

Students might find Parts I-III of this book valuable in appreciating the theoretical propositions of Part IV. The specialist reader, however, might have preferred the relevant facts to have been referenced and integrated into the presentation of the theory. In Vol. 2 of the work (which, I'm told, will not be published until 1988 or so), Livesey proposes to identify the neural systems for directing attention to relevant stimuli and for organizing appropriate behaviour on subsequent encounters, with special reference to the role of the limbic system in the mediation of this learning. □

Eric A. Salzen is Anderson Professor in the Department of Psychology, University of Aberdeen, Old Aberdeen AB9 2UB, UK.

Universal classics

Michael Rowan-Robinson

Cosmological Constants: Papers in Modern Cosmology. Edited by Jeremy Bernstein and Gerald Feinberg. *Columbia University Press:1986. Pp.328. \$38.*

THE twentieth century has seen the most remarkable development in our understanding of the Universe, and Bernstein and Feinberg's idea of collecting together some of the classic papers of the great modern age of cosmology was an excellent one. Most of the really important ones are here. The first tentative speculations by Einstein in 1917 about a homogeneous and isotropic, but static, universe; de Sitter's disturbing revelation (also of 1917) that Einstein's equations allowed the possibility of an expanding universe; Friedmann's spectacular solution of the cosmological problem in general relativity (1922 and 1924), giving us the models which have formed the basis for cosmological studies ever since; Robertson's (1929) elegant reformulation of these solutions with far more satisfactory underlying assumptions. And then there are the observations, as usual limping along behind theory, with Hubble's 1929 statement, on the basis of the flimsiest of evidence, of his immortal law.

There is quite a gap until the hot Big Bang of Gamov (1948) and Alpher and Herman (1949), and then another long gap until those two remarkable papers which appeared back-to-back in *Astrophysical Journal* in 1965: the two-page announcement by Penzias and Wilson of the discovery of the microwave background, with virtually no realization of the significance of the discovery, and the hasty interpretation by Dicke, Peebles, Roll

and Wilkinson, pipped to the discovery by perhaps a year.

Finally there is a batch of papers on the new synthesis of particle physics and cosmology, though it is probably too soon to say that any of the work is on the right lines. We have the insight of Sakharov (1967), in his happier days, on baryon non-conservation, and its realization in grand unified theories by Yoshimura, Weinberg and others; Kibble (1976) on cosmic strings; Preskill (1979) on the monopole problem; and Guth's proposal (1981) of the inflationary universe.

Not all of the papers included are of the same standing as those mentioned above. The editors have chosen quite the wrong one by Sandage on the Hubble constant — a minor 1968 paper, using globular clusters, instead of that of 1956 in which he revised Baade's distance scale by a factor of nearly four to arrive at a Hubble constant of 75. The famous 1941 paper by McKellar, measuring a background temperature of 2.3 K, is absent, as are the near-miss papers of Hoyle and Tayler (1964) and Doroshkevich and Novikov (1964) on helium production, and there is nothing from the ever-influential Zeldovich.

The book has been produced in camera-ready format, so I do not understand why the editors opted to retype every paper rather than reproduce them in facsimile. I think most readers would have been willing to do a bit of work on the notation to get that breath of history. But even if it reads like the ultimate summer school proceedings, this anthology will be irresistible to all with a serious interest in cosmology and the history of science. □

Michael Rowan-Robinson is Reader in Astronomy in the School of Mathematical Sciences, Queen Mary College, Mile End Road, London E1 4NS, UK, and author of *The Cosmological Distance Ladder* (W.H. Freeman, 1985).

Uncertainty in science: is the giant panda a bear or a raccoon?

Ernst Mayr

The few remaining doubts that the giant panda is a bear depend on the belief that it ought to be closely related to the lesser panda, and on controversial interpretations of molecular sequence differences.

PHILOSOPHERS of science have written volumes on the method of science and on the certainty it produces when properly applied. In practice, scientific progress is often embarrassingly halting, uncertain, and unpredictable. That scientists are often slow in accepting new theories, no matter how well substantiated, has been excellently documented by Barber¹. H. J. Muller angrily exclaimed in 1959: "A hundred years without Darwin are enough", yet for the world outside evolutionary biology, Muller's complaint still holds today. More interesting are cases where some scientists refused to accept a theory considered to be abundantly proven by others. This is particularly often the case when the evidence consists of observations that can be interpreted in different ways depending on the conceptual framework of different scientists. An apt illustration for this situation is the old question about the relationship of the giant panda: is it a bear or is it, like the lesser panda, a member of the raccoon family?

When the giant panda was discovered in 1869, it was described as a new species of bear (*Ursus*)² but in the very next year another zoologist claimed that the giant panda was not a bear at all, but rather a relative of the lesser panda (*Ailurus*), a member of the raccoon family (Procyonidae), and he placed the giant panda in the genus *Ailuropoda*³. From that date on, for almost a century, zoologists argued about to which family the giant panda or 'bamboo bear' belonged. During this period 19 authors opted for bear, and 18 for raccoon. (I have ignored those authors who, in order to dodge the issue, raised *Ailuropoda* to the rank of a separate family without committing themselves as to whether it was closer to the bears or to the raccoons, and a few papers in which the authors clearly reveal an ignorance of the literature and of the issues.)

The period of uncertainty seemingly ended in 1964 when D. D. Davis published a superb comparative anatomical study of the giant panda, in which he showed for characteristic after characteristic that this animal is clearly a bear adapted for his peculiar food niche⁴, which, as everyone knows, is an almost exclusive diet of bam-

boo. S. J. Gould⁵ has recently referred to Davis's monograph as "our century's greatest work of comparative anatomy". What distinguished Davis's analysis were two features: he based his conclusions on a detailed analysis of a very large number of anatomical characteristics of the giant panda and his putative relatives and, after having arrived at the conclusion that it was a bear, he showed what selection pressures must have been responsible for the seeming similarities to the lesser panda. In this work Davis demonstrated the first application of his new concept of evolutionary morphology⁶. This consists of an attempt to reconstruct the selection pressures leading to a deviation from the homologous features of the ancestral species. (Ewer in a review of Davis's monograph accused Davis of bias in favour of the bear relationship and inadequate consideration of possible raccoon relationship. After carefully going through Davis's account I am quite unable to discover any bias. After having established the overwhelming evidence in favour of bear relationship Davis, in classical hypothetico-deductive fashion, tested all seeming similarities with the lesser panda to determine whether they could be explained as a consequence of a shift to a harsh vegetarian diet. This I do not consider bias.)

One would think that a presentation of such overwhelming evidence for the bear relationship of the giant panda would end the argument. But this was not to be the case. A few zoologists continued to favour a relationship of 'the two pandas'. There were two 'accidents of history' which seemed to support such a relationship. First, both species occur in the same general area, southwestern China. If the lesser panda is a raccoon, and not related to the giant panda, then all of its near relatives would be in the Americas, since there is not a single other living raccoon relative in the Old World. Such a distribution seemed improbable. Second, the vernacular names lesser panda and giant panda lead one almost automatically to assume closest relationship of these two animals. It is interesting to note that in Germany, where the giant panda is often

referred to as *Bambus Bär*, a much higher percentage of specialists opted for the bear relationship than in the English-speaking countries. Of the nineteen pre-1967 authors listed by Thenius⁷ as favouring the bear relationship, six are German-speaking, while there is not a single German-speaking author on his list of the 18 authors who favoured a raccoon relationship.

Davis's conclusions were so convincing that they were accepted almost universally⁸⁻¹¹. The only dissent came from a number of students of behaviour¹²⁻¹⁵. Their reasons for a belief in raccoon relationship were stated most completely and most forcibly by Ewer, in her authoritative handbook, *The Carnivora*¹³. Why was she so convinced of the close relationship of the two pandas? Reading between the lines, one feels that it was her observation of the similarity in the feeding behaviour of the two pandas, as studied in London Zoo, that convinced her. She was startled to see that the lesser panda (*Ailurus*) was holding plant stems in its paws exactly like the giant panda, and concluded that this was the starting point of all the specializations of the giant panda, including the 'panda's thumb'. Ewer, an outstanding student of behaviour, evidently considered the similarity in feeding behaviour of the two pandas as a decisive argument in favour of their relationship. This was her base line, and the evidence provided by other characteristics was meaningful only given this foundation. The similarity of feeding habits and the correlated adaptations of teeth and jaw musculature convinced her that the pandas were closely related. However, her basic assumptions can be challenged. It is now believed that the behaviour elements having to do with feeding are among the most flexible of all¹⁷. Furthermore, the giant panda is not nearly as different in diet from the bears as is sometimes implied. Bears are basically omnivorous, and even such seemingly pure carnivores as polar bears and brown (grizzly) bears shift at certain seasons to a diet of berries. Some of the tropical bears are highly specialized in various directions. For instance, the honey

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

bear (*Melursus*) "is a specialized eater of insects, honey, and soft fruits and [has] peculiar modifications of lips, tongue, and incisors in adaptation to this diet"¹³. So why should not the giant panda's masticatory peculiarities also be an adaptation to its specialized diet? Indeed, Davis showed how easily the morphology of *Ailuropoda* could be derived from a more generalized bear type. Ewer, curiously, was so convinced of the correctness of her interpretation that in much of her account she simply compared "the pandas" with "the bears", meaning *Ursus*.

Ewer's actual evidence in favour of the relationship of the pandas was not very convincing. Although she emphasized their similarity in dentition (premolars) and massiveness of the skull, she also admitted that the equally vegetarian South American bear *Tremarctos* shows "a strong resemblance to the giant panda". Davis had quite properly focused on the evidence of the jaw muscles and dentition as the most unbearable features of the giant panda and had shown that these adaptations are precisely what one would expect in a bear that had shifted from an omnivorous to a strictly vegetarian diet.

One of the most characteristic specializations of the giant panda is its famous thumb¹⁶. This induced Ewer to say¹³: "It is necessary to account for the presence of the enlarged radial sesamoid forming the panda's thumb. A lesser panda grips a stem in almost exactly the same way as does the giant panda and one has only to watch him doing so to see how advantageous the development of the extra thumb would be: a bear's use of its paws, on the other hand, does not suggest why the sesamoid should ever have become enlarged". Actually this mode of feeding did not result in the development of a thumb in the lesser panda. Behaviour is almost invariably the pacemaker in the development of morphological novelties¹⁷, and there is no reason why the large bears (*Ursus*) with their entirely different feeding habits should develop a sesamoid.

The remainder of Ewer's evidence is even less convincing. She pointed out that

the giant panda has anal scent glands and marks its territory, a habit apparently absent in the large bears. However, this marking habit, a widespread feature in related families, is a primitive characteristic that was lost in *Ursus*. The absence of a primitive characteristic is, of course, a poor indicator of relationship. Furthermore, I know of no good studies of this feature in the tropical bears. Ewer also claimed for the giant panda a "very different structure of the reproductive organs"¹³, but the reputed similarity between the two pandas in the structure of the genitalia has since been shown to be an error⁸. She also emphasized a difference between the giant panda and the northern bears with respect to hibernation. This is, of course, a strictly adaptive feature and I know of no evidence that any tropical or subtropical bear hibernates. As Schaller has shown¹⁵, the nutritive value of bamboo is so low that it would not enable the giant panda to store up sufficient fat to permit hibernation. Even raccoons have periods of quiescence when wintering in cold temperate regions. Finally, Ewer pointed out that the chromosome number is smaller in the two pandas than in the large bears (*Ursus*). This is a notoriously inconclusive characteristic in a comparison of higher taxa, and since the giant panda is the only living representative of an entire subfamily of bears, its chromosome number might well be the same as that of other, now extinct, bears. Indeed, as has since been shown by O'Brien¹⁸, the chromosomes of the giant panda are remarkably similar to those of bears.

When one looks at Ewer's evidence critically, not a single item remains that favours the relationship of the giant panda with the lesser panda over that with the bears. O'Brien has recently shown in another case how careful one has to be in the use of behavioural evidence. The cheetah (*Acinonyx*), owing to its adaptation for running, has always been considered the most aberrant of all cats, but a study of immunological distances¹⁹ has shown that the cheetah is quite closely related to the lion-tiger group of cats, with many other species of cats being far more distant.

All new investigations published after 1970 produced additional evidence for the bear relationship of the giant panda. A few early molecular studies firmly associated the giant panda with the bears²⁰⁻²². The study of the fossil evidence was particularly revealing. It showed that fossil bears clearly fall into two groups, one giving rise to the large bears as we know them, the other branch giving rise to a number of genera and species that have since become extinct, leaving the giant panda as the last survivor^{7,23,24}. Two of these extinct bears (*Agriotherium* and *Indarctos*) connect the giant panda with

the more typical bears. The study of the fossil record was also important in calling attention to the diversity of bears. For a worker in the temperate zone of the Northern Hemisphere, the word bear brings to mind such species as the polar bear, brown bear and black bear. But some rather different bears exist in the subtropics and tropics of southeast Asia, and a very aberrant one, the spectacled bear (*Tremarctos ornatus*) in South America. A Pleistocene radiation produced a group of small dog-sized bears (*Ursavus*) that was not only ancestral to the living bears, but also includes one particular species (*U. depereti*), which has most of the features one would expect in the ancestor of the giant panda⁷. The fossil record of the lesser panda is equally revealing²⁴. The Procyonidae already showed considerable heterogeneity in the Miocene, foreshadowing the great diversity of the living survivors. Raccoon, coati, kinkajou, and lesser panda are quite different from each other, and could well be divided into several different families or subfamilies, as some authors have suggested. The fossil evidence also shows that the lesser panda, through the Miocene genus *Sivanasua*, is much more closely related to the other procyonids than it is to the bears (including the giant panda). The fossil record provides no evidence for any connection between raccoons and the giant panda. Thenius summarized his extensive studies of the anatomy and fossil history of the bears by emphasizing that "relationships between giant panda and lesser panda do not exist". The evidence from these diverse areas seems so compelling that all authoritative handbooks or lists of mammals pub-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

... Distantly to the giant panda.

lished in that period accepted the bear relationships of the giant panda without comment⁸⁻¹¹. Only some of the behaviour students were not yet persuaded^{14,15}.

To settle the argument once and for all, it was therefore legitimate to make another attempt to test the relationship of the giant panda, using the modern methods of molecular and chromosomal analysis. Sibley and Ahlquist²⁵ have recently shown how valuable the molecular approach can be, by working out an entirely new cladogram of avian relationships. O'Brien and associates, who made the startling discovery of the close relationship of the cheetah (*Acinonyx*) to the lion-tiger group, undertook a modern analysis of the giant panda problem¹⁸. They unnecessarily overdramatized the issue by referring to "the riddle of the giant panda's phylogeny" (it really was no longer much of a riddle by 1985), and by referring to the lesser panda as "the diminutive Chinese relative of the giant panda", when by that time virtually no one except for Ewer and a few other behaviour students still believed in that relationship. Be that as it may, the diversified approaches by O'Brien and his collaborators support the findings of the anatomists (particularly Davis) and of the palaeontologists (Thenius, Hendey) so massively that any doubt on the bear relationship of the giant panda can no longer be maintained. A number of separate techniques, DNA hybridization, isozyme genetic distance, immunological distance, and the karyological evidence all result in a nearly identical phylogenetic tree, in which the giant pandas diverged from the other bears only about 20 million years ago, while the bears and raccoons separated at least 40, but perhaps even 50 million years ago. The lesser panda branched off the procyonid lineage quite early, perhaps soon after it had split off from the bear branch.

One might have thought that this was the end of the story, but hardly was the O'Brien paper off the press when a paper was published by a group of well known protein analysts showing that the haemoglobins of the two pandas differ by far fewer substitutions than do those of the giant panda from the bears or the lesser panda from the raccoons. The haemoglobins of the two pandas show a minimum mutation distance of seven amino acids, while giant panda and polar

bear differ by a minimal mutation distance of 11, and lesser panda and raccoon by a distance of 18 mutations. The view was therefore revived that the two pandas are, after all, each other's closest relatives²⁶.

It would seem that this suggestion is clearly refuted by the near-unanimous and highly diverse evidence in favour of the bear relationship of the giant panda recorded above. Instead it would seem to be another instance of so-called mosaic evolution, that is, divergent evolution of different components of the genotype. Evidently the haemoglobins studied by Tagle *et al.* have evolved at a different rate and in a different direction from the molecules studied by Sarich²¹ and by O'Brien¹⁸. There is now little question that much molecular change during evolution is near neutral and the possibility of the existence of a 'molecular clock' largely depends on this property of most molecular changes. However, there are other molecules in which the replacement or conservation of base pairs is largely controlled by selection. A good example of such conservation is the near identity of human and chimpanzee haemoglobins α and β . The two species are separated by 10-16 million years of evolutionary history, sufficient time for these molecules to be drastically restructured, given the known high frequency of haemoglobin mutations in the human species. The fact that the haemoglobins are still nearly identical shows how powerful the selection forces on haemoglobin are. In the case of man and chimpanzee this influence has been conservative, but in the case of the bears it might well have been centrifugal.

One must know much more about the DNA sequence of the proteins of other bears, particularly *Tremarctos*, and of other Procyonidae, before one can say which haemoglobins are ancestral and which are derived. Two alternatives are most probable: (1) The giant panda still has a rather widespread ancestral haemoglobin, sharing most of it with the lesser panda and other procyonids, but the *Ursus* group of bears has diverged in a novel direction. In that case it would be possible that *Tremarctos*, the most aberrant of the Ursinae, is still somewhat intermediate, or (2) The two pandas, living in the same geographical area and both having switched from an omnivorous to a

strictly vegetarian diet were exposed to very similar selection pressures which somehow favoured a convergent evolution of their haemoglobins. The probability of this hypothesis is strengthened by the fact that other instances are known of a response of haemoglobin to selection pressures. The sickle-cell haemoglobin in Man at once comes to mind. Braunitzer's group has also discovered cases of adaptive haemoglobin changes in birds. The bar-headed goose (*Anser indicus*), which nests in Tibet and flies 9,000 m high when crossing the Himalayas on the way to its winter quarters in India, has haemoglobin changes compared with its relatives that facilitate oxygen uptake²⁷. At least one vulture which flies at the same altitudes has a similar haemoglobin change (G. Braunitzer, personal communication).

Which of the various explanations is correct cannot be decided until we know much more about the haemoglobins of the relatives of the two kinds of pandas. To determine the cause of the similarity in their haemoglobins is certainly a fascinating evolutionary problem. If the haemoglobins have become so similar through adaptive convergence, as seems highly probable, it reinforces the claim of leading taxonomists that all taxonomic characters should be carefully weighted. Dendrograms based on unweighted differences can be highly misleading.

Let us now go back to the question asked at the beginning of this paper. Why is there so much uncertainty in those branches of science in which observation provides the major material for scientific theories? In the case of the relationship of the giant panda we have seen that the very same evidence was evaluated very differently by anatomists and by students of behaviour. There was no other way to determine which of the two conclusions was correct except by providing additional data and analysing each piece of evidence more critically. When this was done, everything fell into place. One can feel reassured in one's belief that proper evaluation and testing will ultimately lead to secure conclusions, even when based on observational evidence, and even where experimental testing is unavailable.

Ernst Mayr is at the Museum of Comparative Zoology, The Agassiz Museum, Harvard University, Cambridge, Massachusetts 02138, USA.

1. Barber, H. *Science* **134**, 596-602 (1961).

2. David, A. *Nouv. Arch. Mus. Hist. nat., Paris* **5**, 12-18 (1869).

3. Milne-Edwards, A. A. *Sci. nat. Zool. Ser.* **5** art 10, 1. *C.R. Hebd. Seanc. Acad. Sci. Paris* **70**, 341-342.

4. Davis, D. D. *Fieldiana, Zool. Mem.* **3** (Chicago Nat. Hist. Museum, 1964).

5. Gould, S. J. *Discover* **7**, 40-48 (1986).

6. Mayr, E. *Suppl. Vol. Dict. Sci. Bio.* (Scribner, New York, in the press).

7. Thenius, E. *Z. Säugetierk.* **44**, 286-305 (1979).

8. Chorn, J. & Hoffmann, R. S. *Ailuropoda melanoleuca* in *Mammalian Species*, No 110 (Amer. Soc. Mammalogists, 1978).

9. Starck, D. in *Vergleichende Anatomie der Wirbeltiere*, p. 217 (Springer, Berlin, 1978).

10. Honacki, J. H., Kinman, K. E. & Koepl, J. W. in *Mammal Species of the World* (Allen Press, Lawrence, Kansas, 1982).

11. Anderson, S. & Jones, J. K. Jr. in *Orders and Families of Recent Mammals of the World* (Wiley, New York, 1984).

12. Morris, D. & Morris, R. *Men and Pandas* (Hutchinson, New York, 1979).

13. Ewer, R. F. *The Carnivora* p. 393 (Cornell University Press, Ithaca, New York, 1973).

14. Eisenberg, J. F. *The Mammalian Radiations* (University of Chicago Press, Chicago, 1981).

15. Schaller, G. B. *The Giant Pandas of Wolong* (University of Chicago Press, 1985).

16. Gould, S. J. *The Panda's Thumb* (W. W. Norton, New York, 1980).

17. Mayr, E. in *Evolution and the Diversity of Life*, 694-711 (Harvard University Press, Cambridge, 1976).

18. O'Brien, S. J. *et al. Nature* **317**, 140-144 (1985).

19. Collier, G. E. & O'Brien, S. J. *Evolution* **39**, 473-487 (1985).

20. Leon, C. A. & Wiens, A. L. *J. Mammal.* **37**, 11-23 (1956).

21. Sarich, V. M. *Nature* **245**, 218-220 (1973).

22. Sarich, V. M. *Trans. Zool. Soc. London* **33**, 165-171 (1976).

23. Hendey, Q. B. A. S. *Afr. Museum* **81**, 1-109 (1980).

24. Van Valen, L. M. *Nature* **317**, 428 (1985).

25. Sibley, C. G. & Ahlquist, J. *Curr. Orn.* **1**, 245-292 (1983).

26. Tagle, D. A., Miyamoto, M. M., Goodman, M., Hofmann, O., Braunitzer, G., Goeltenboth, R. & Jalkanen, H. *Naturwissenschaften* **73**, 511-514 (1986).

27. Hiebl, I., Schneegans, D. & Braunitzer, G. *Biol. Chem. Hoppe-Seyler* **367**, 591-599 (1986).

Ozone and nitrogen dioxide changes in the stratosphere during 1979–84

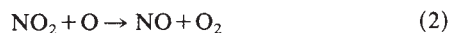
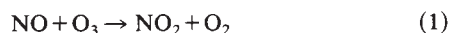
Linwood B. Callis* & Murali Natarajan†

* Atmospheric Sciences Division, NASA-Langley Research Center, Hampton, Virginia 23665, USA

† SASC Technologies, Inc., Hampton, Virginia 23666, USA

Analyses of stratospheric nitrogen dioxide distributions as measured by four different satellite experiments indicate mid-latitude increases of up to 75% during the 1979–84 period. These increases are attributed to enhanced upper atmospheric formation of odd nitrogen during solar cycle 21 with downward transport to the stratosphere. The increases in NO₂ provide an explanation for the recently observed dramatic springtime minima in the Antarctic ozone and suggest the reason for the reported mid-latitude stratospheric ozone decreases observed by satellite and ground-based stations since the mid 1970s.

STRATOSPHERIC NOY (defined as NO + NO₂ + HNO₃ + NO₃ + 2 × N₂O₅ + HNO₄ + ClONO₂) has been of great interest since it was found that its catalytic destruction of O₃ through reactions (1) and (2) is one of the dominant factors in the maintenance of stratospheric O₃ (ref. 1).

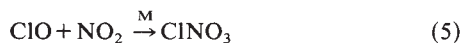


With the recent availability of satellite-based O₃ and NOY species measurements, the temporal and spatial coverage of the data base for some of the NOY constituents has been greatly extended and the uncertainties reduced. It is this collective database that we use to examine the variations of NOY and O₃ during the 1979–84 period. These data include infrared emission measurements of O₃, NO₂, HNO₃, H₂O and T from the limb infrared monitor of the stratosphere (LIMS) experiment, NO₂ solar occultation measurements from the stratospheric aerosol and gas experiments (SAGE and SAGE II), and visible light spectrometer measurements of NO₂ from the SME experiment. Descriptions of these experiments have been published^{2–5} and the data validated.

The distribution in latitude and altitude of NOY as inferred from the LIMS data for October and December 1978 and March and May 1979⁶ indicate peak NOY mixing ratios of 25–27 parts per 10⁹ by volume in the 35–37 km region at mid and low latitudes. Such NOY levels are 20–40% larger than contemporary models predict^{7,8}. It is of considerable interest to resolve this discrepancy as NOY levels significantly affect O₃ balance calculations through reactions (1) and (2) and determine the extent to which growing levels of atmospheric chlorine will destroy atmospheric O₃ by catalysis^{8,9} in reactions (3) and (4).



Increasing levels of NOY slow this Cl_x catalytic destruction due to reactions (5) and (6).



The importance of the interactions that exist between atmospheric O_x–NO_x–Cl_x–HO_x has been well documented⁹.

Figure 1 illustrates the monthly averaged LIMS and SME

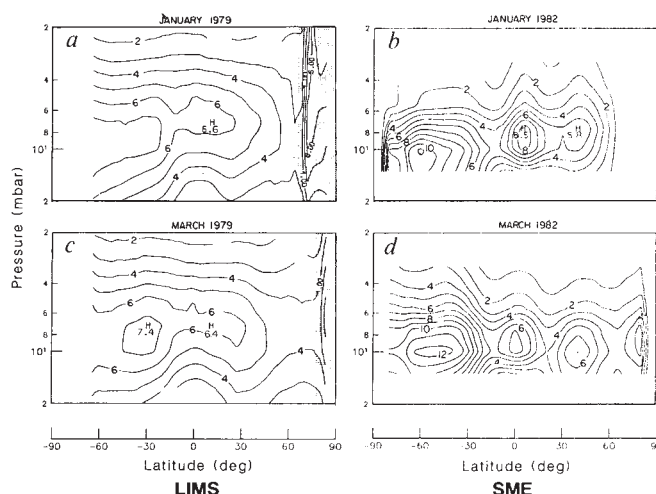


Fig. 1 Comparisons between 1979 and 1982 monthly mean day-time NO₂ mixing ratios as measured by the LIMS experiment aboard the Nimbus 7 spacecraft and the visible light spectrometer aboard the SME spacecraft. Local measurement times for the LIMS experiment range from 1:10 to 3:33 pm at 20° and 60° S, respectively. Local measurement times for the SME NO₂ observations are near 3:00 pm. LIMS NO₂ observations are well distributed in longitude (with little variation) while SME measurements are collected each day in four orbits between 40° W and 140° W. No significant diurnal corrections are required for direct comparisons of LIMS and SME data at 55° S.

NO₂ distributions for January and March in 1979 and 1982, respectively. We note that at 55° S these two sets of measurements are made at the same local time, 3:00 pm. Therefore, at that latitude and those closeby, no diurnal corrections of the NO₂ measurements are required for direct comparisons of these satellite data sets to be made. The results of the comparisons are rather startling. In a general sense the distributions are similar with maximum levels of NO₂ occurring in both data sets at the 7 to 10 mbar level and with a maximum appearing in the Southern Hemisphere. Otherwise, there are significant differences. Maximum values (in the Southern Hemisphere) as determined from the LIMS instrument range from 6.2 to 7.4 parts per 10⁹. For the SME measurements in 1982, maximum values range from slightly more than 11 to more than 12 parts per 10⁹ by volume with the maxima occurring at higher latitudes. At the maxima, this represents an increase in NO₂ mixing ratios of 50 to 70% in a period of three years.

To determine the significance of these differences, we have examined the published error budgets for both the LIMS and

SME NO_2 measurements^{2,5}. For the LIMS NO_2 data, the root sum square error at 30 km (approximately 10 mbar) is $\pm 23\%$ with a random component of approximately 7% (J. M. Russell, personal communication) being introduced due to errors in the temperatures used in the retrieval algorithm. For the SME data, the published error analysis indicates a root mean square (r.m.s.) error at 28 km and 38 km of $\pm 21\%$ and $\pm 65\%$, respectively. These estimates include a random component at 28 km of $<5\%$ and at 38 km of $<25\%$. Accounting for the reduction of the random component of the estimated error that accrues when many profiles are averaged over monthly periods, the NO_2 changes for January and March at 10 mbar and at latitudes near 55°S are substantially outside of the error bars. The same is true for the February LIMS and SME data comparisons (not shown).

In an effort to confirm these startling differences in measured NO_2 , archive NO_2 data from SAGE and preliminary NO_2 data from SAGE II (M. P. McCormick, personal communication) were examined. Comparisons of SAGE and SAGE II data are shown on Fig. 2 for comparable southern latitudes and at times (near the end of November and in early December) as near one another as the orbital coverage of these satellites allowed. Both SAGE and SAGE II measure the NO_2 in solar occultation, and the measurements are made with essentially the same technique. For SAGE II, one additional channel was used for the NO_2 measurements providing better accuracy. Given the close similarity of the two experiments, direct comparisons of these data sets can be made.

From the isoline plots for the 1979 and 1984 data (Fig. 2a, b) several similarities are apparent. The maximum mixing ratios for both data sets occur near 32 to 33 km and the local maxima of NO_2 at this altitude occurs in both data sets at longitudes centred at 45°E and 250°E . Most interesting, however, is the magnitude of the local maxima. For the 1979 SAGE data, maxima range from 6.8 to 7.0 parts per 10^9 . For the 1984 SAGE II data, maxima in excess of 11 parts per 10^9 by volume are shown, an increase of 60% from 1979 to 1984.

Figure 2c shows a comparison of zonal averages for several days of SAGE and SAGE II data for comparable latitude bands and times. The error bars shown are based upon the published SAGE error budget³ with the random component of the error reported by these authors reduced by the factor $\sqrt{N}$ where N is the number of profiles used in the average (approximately 15 profiles per day). Error bars on the SAGE II data are conservatively taken to be those for the SAGE experiment.

Results shown in Fig. 2c indicate that at the level of the maximum mixing ratios, the NO_2 has increased by 75% from 5.7 to 10 parts per 10^9 between 1979 and 1984. The changes indicated between 25 and 40 km are well outside the indicated error bars for the different altitudes.

We are confronted with a rather dramatic finding. Analysis of four sets of satellite data indicate that from 1979 to 1982–84 stratospheric NO_2 mixing ratios have increased from 38 to 75% at mid-southern latitudes and an altitude range of at least from 25 to 40 km. Smaller increases are observed over the equatorial latitudes and in the Northern Hemisphere. Using the SME data at 55°S and 10 mbar, we have inferred the NOY using previously described techniques⁶. For March 1982 at 55°S and 10 mbar the NOY levels inferred are 33 parts per 10^9 compared to 20 parts per 10^9 calculated for the same month, latitude and altitude using the 1979 LIMS data. This represents a 65% increase in NOY.

In searching for still further confirmation of stratospheric NOY increases, we have examined column sum data for NO_2 for levels between 20 and 35 km (ref. 10) taken at Scott Base (77.8°S) during September and October 1982. The data indicate a seasonal growth in column NO_2 from 1×10^{15} molecules cm^{-2} at the end of August to about 5×10^{15} to 6×10^{15} molecules cm^{-2} by the third week in October. In a detailed analysis¹¹ of the spring minimum in antarctic O_3 and the related NO_x – O_x – Cl_x –

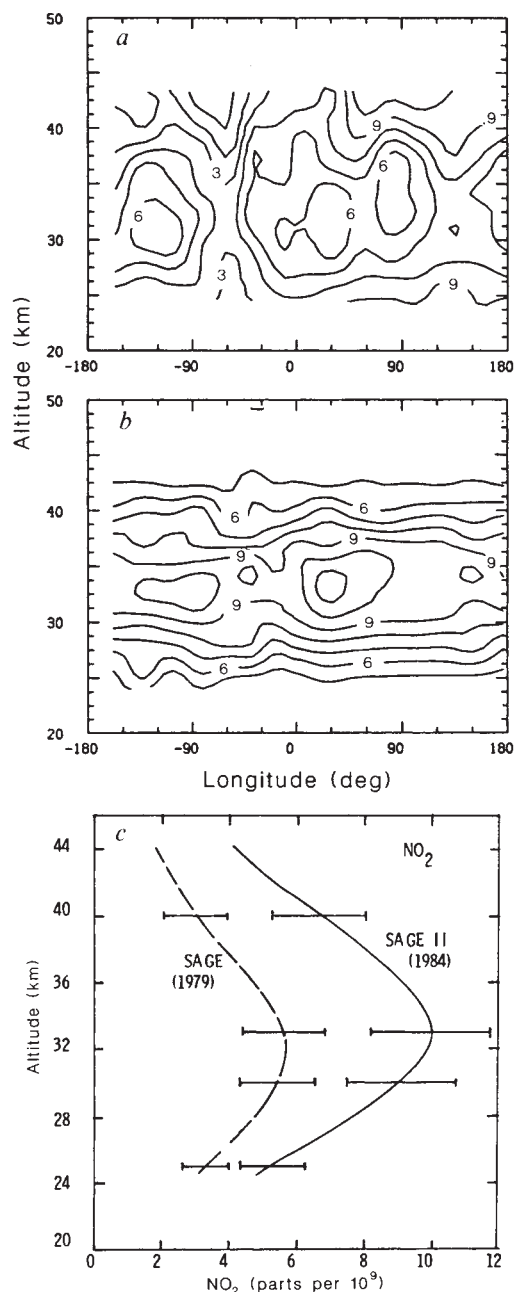
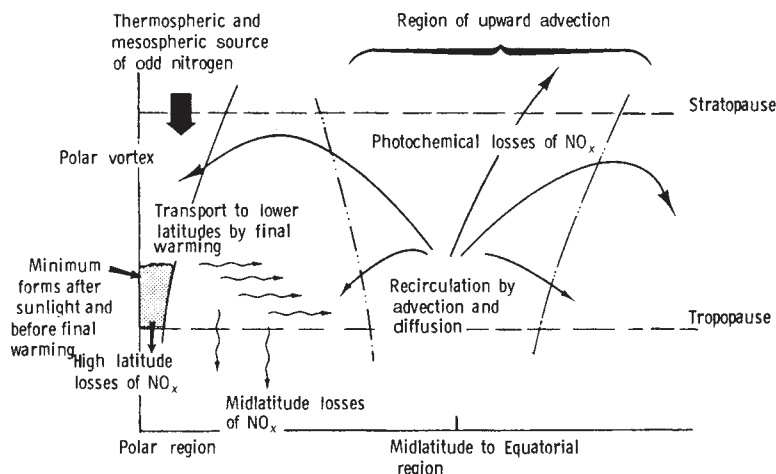


Fig. 2 Solar occultation measurements of daytime mixing ratios of NO_2 (parts per 10^9) by the SAGE and SAGE II (preliminary data) satellites. *a*, Isolines plot of 1979 SAGE data for 25 November 1979. Profiles used for this figure were measured over a latitude range of 65.3°S to 66.9°S . *b*, SAGE II NO_2 isoline distribution for 4 December 1984, for the latitude range 63.6°S to 66°S . *c*, Comparison of zonally averaged SAGE and SAGE II data. SAGE data in this panel is the average of all data for the days 25 November to 27 November 1979, and covers a latitude range of 61°S to 66°S . SAGE II data in this panel is the average of data for 28 November to 4 December 1984, and covers a latitude range of 63.8°S to 67.6°S . Error bars shown are as published³. This is a conservative estimate for SAGE II. (Data for SAGE II provided by Drs M. P. McCormick and W. P. Chu, personal communication).

HO_x chemistry which occurs, we have shown that these NO_2 column sum measurements are photochemically consistent with local NOY levels of 32–35 parts per 10^9 . Consequently these column data are also consistent with the approximately 33 parts per 10^9 of NOY inferred from the SME and SAGE II data for the 1982 and 1984 periods. We conclude that these large stratospheric increases of NO_2 (and hence NOY) are real.

Fig. 3 Schematic illustration of the buildup of stratospheric NOY. NOY produced in the thermosphere and mesosphere during solar cycle 21 is advected downward into the stratosphere in the winter hemisphere. Further advection to mid stratospheric levels occurs principally in the Southern Hemisphere within the polar night vortex. During the seasonal transition there is rapid quasi-horizontal irreversible mixing to lower latitudes. Recirculation throughout the stratosphere occurs due to upward, then poleward and downward, advection and also by further mixing due to transient planetary wave activity. There are significantly higher levels of wintertime transient planetary wave activity in the Northern, compared to the Southern, Hemisphere in the stratosphere and lower mesosphere. As a result of the Northern Hemispheric mesospheric latitudinal mixing associated with this transient wave activity, there is less NOY advected into the Northern Hemisphere stratosphere than in the Southern Hemisphere. Once the NOY is in the northern polar vortex, a nearly continuous mixing out occurs due to transient episodes which occur throughout the winter²⁵. This explains the absence of pronounced O₃ minima in the Northern Hemisphere.



Source of odd nitrogen

It is generally accepted that the sources of NOY in the stratosphere are the reaction within the stratosphere of



with contributions by the downward transport of NOY from the thermosphere and mesosphere. The latter contribution occurs principally during the polar night^{12,13}. There are also contributions to the stratospheric NOY production by galactic cosmic rays, solar proton events, and relativistic electron precipitation¹². There may be some transport into the stratosphere from lightning sources in the upper troposphere¹⁴, primarily in the equatorial latitudes. In the current model formulations, however, the principal source is reaction (7).

We consider the possibility that there may be larger than expected downward transport of NOY from the lower mesosphere and upper stratosphere. Measurements of nighttime NO₂ within the stratosphere. It is known that the global average column production of NOY (in the form of atomic nitrogen) above 100 km is more than two orders of magnitude larger than the NOY production in the stratosphere due to reaction (7)¹⁵. The possibility that thermospheric NOY can be transported to the stratosphere has been investigated by a number of authors^{13,16-18}. Results indicate that such transport would have to occur primarily during the polar night to avoid significant loss initiated by photodestruction of NO.

It is important to note that published observations provide convincing evidence of downward transport to the lower mesosphere and upper stratosphere. Measurements of night-time NO₂ by the LIMS experiment suggest levels of NOY of 175 parts per 10⁹ during 5-9 January, 1979 at 60-65 km and at 80° N and 240-300° E¹⁹ (within the polar vortex) with indications of downward transport to the upper stratosphere. Rocket measurements of NO made in February 1985 at 65° N confirm NO mixing ratios of 149 parts per 10⁹ at 52 km (ref. 20). Analysis of NO gamma-band fluorescence features using solar backscatter ultraviolet (SBUV) ultraviolet radiance data in the spectral scan mode²¹ indicates the presence of column abundance of NO of 1×10^{15} – 2×10^{15} molecules cm⁻² above 1 mbar near the Southern Hemisphere winter terminator on 20 June and 24 July 1979, and 12 June and 30 July 1980. The same technique suggests column values of 1×10^{15} molecules cm⁻² near the winter terminator and above 1 mbar in the Northern Hemisphere for 3 and 6 December 1979, and for 17 and 27 December 1980. These are significantly high levels. For comparison we note that the column sum of LIMS measured night-time NO₂ above 20.5 km at 20° N during 5-9 January 1979 and for 240° E to 300° E was determined²² to be 4.5×10^{15} molecules cm⁻².

The fact that high levels of NOY are located in the polar winter latitudes in the lower mesosphere and upper stratosphere is consistent with a thermospheric source. Given that it is well known that climatological advective descent occurs within the polar vortex during the polar night^{11,13,18,23}, transport of the above-mentioned high levels of NOY into the stratosphere is essentially guaranteed. With descent rates (during June and July at high southern latitudes) of approximately 1 km per day near the stratopause, 0.6 km per day near 40 km, and 0.1 km per day near 30 km, this NOY descent is consistent with the previously mentioned high levels of NO₂ found at mid to high southern latitudes. It is also consistent with levels of column NO₂ found¹⁰ subsequent to the Austral winter, within and near the southern polar vortex before the final warming. The path and timing of this advective descent have been confirmed with diabatically diagnosed transport and trajectory calculations^{11,13,18,23}.

In summary, the individual pieces of observational and theoretical evidence are as follows. Very high levels of NOY have been observed in or near the polar winter night at the level of the stratopause or within the lower mesosphere in both hemispheres¹⁹⁻²¹. Transport calculations indicate that strong downward advective transport of NOY into the stratosphere should occur within the polar night vortex^{11,12,18,23}. High levels of NO₂ have been observed within the 20-35 km level of the southern polar vortex shortly after the southern spring equinox and before the final warming¹⁰. Analysis of satellite data and three-dimensional model calculations indicate the presence of relatively rapid quasi-horizontal irreversible mixing associated with strong transient wave activity and with the winter to spring dynamical transition resulting from the final warming²⁴⁻²⁶. Finally, NOY distributions inferred from LIMS data in 1978 and 1979 are consistent with such horizontal transport associated with the seasonal transition in the Southern Hemisphere in 1978 and with the reestablishment of downward transport of NOY during the Austral fall⁶.

Based upon the previously reported supporting observational and theoretical evidence and the presently reported significant differences in the NO₂ observations for the 1979 to 1984 period, we conclude that the increasing levels of NO₂ which have been observed in the stratosphere have as their origin the mesosphere and probably the thermosphere. We further suggest that they are due to increased thermospheric and mesospheric production of NOY during solar cycle 21 (the second most active in 250 years) which reached a sunspot maximum in late 1979. It is well known that thermospheric NOY production (principally in the form of NO) is significantly increased during solar maximum conditions by several processes. Thermospheric and mesospheric NOY concentrations are, therefore, expected to be higher during such maximum periods^{18,27}.

The mechanism by which this buildup of NOY may occur in the stratosphere is summarized schematically in Fig. 3. The individual and key elements of this mechanism have each been discussed in the scientific literature referred to above. They are the advective descending motion in the polar night, the rapid quasi-horizontal irreversible mixing associated with the seasonal transition (and strong transients) and the recirculation and irreversible mixing of the NOY within the stratosphere after the seasonal transition.

During this buildup of NOY in the stratosphere, no NOY losses occur during downward transport within the vortex during the polar night. At lower latitudes, the usual NOY losses from the stratosphere and above occur by two processes. The first is photochemical and is governed by the following reactions:



The lifetime of a column of NOY above 20 km against loss by the above reactions within the stratosphere is 10 years at 30° N in May and 28 years at 60–70° N. The second loss process is through downward transport to the troposphere at mid and high latitudes, principally in the form of NO₂, HNO₃ and N₂O₅. The calculated stratospheric lifetime of NOY against these combined loss processes is 3–4 years. With such lifetimes, and with a large infusion of NOY from the mesosphere during the 1979 to 1984 time period, stratospheric levels of NOY must grow. This is what the observations clearly indicate. Based upon this mechanism, we would expect NOY levels to begin to increase 1–2 years after solar minimum conditions (which occurred in late 1975 for the beginning of solar cycle 21) and to continue increasing through solar maximum (late 1979) and for 3–4 years afterwards.

The extent to which there are significant increases in NOY during a solar cycle depends on how active that particular cycle happens to be and on the nature of the solar activity relative to the formation of NOY. In terms of sunspot number, solar cycle 21 had a maximum 60% higher than the mean of cycles 8–20²⁸. In February and March of 1982, 2½ years after maximum conditions, the sunspot number average was 158, significantly higher than the monthly average of 135 which occurred at the previous maximum in 1969. Also, based on the observations of NOY species at or near the polar winter stratopause and lower mesosphere, solar cycle 21 maximum activity was such that a large production of NOY resulted. Before the present cycle, one must go back to near the maximum of solar cycle 19 (1957–58) to find monthly averaged sunspot values as large or larger.

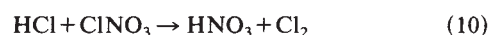
Stratospheric effects of odd nitrogen increases

Springtime minimum in Antarctic ozone. Given the large NO₂ increases observed by satellite in the mid stratosphere, and the high mixing ratios of NOY that the column measurements of NO₂ during the Antarctic spring imply, we consider whether or not they are related to the recently reported Antarctic O₃ hole²⁹. To do this we apply our photochemical code³⁰ with the recently recommended rates³¹ to the calculation of species variations along predetermined meridional trajectories calculated from an advective transport field. This transport field has been diabatically diagnosed from a 12-month climatology of recent satellite measurements of T, O₃ and H₂O^{11,23}. The limitations associated with the use of such transport within the relatively stable southern polar vortex are not a serious problem in the present study¹¹.

The meridional advective trajectories were run backward in time to establish the advective history of the air mass within which the O₃ reductions occur. Below 20 km, reverse trajectories indicate the source region to be 50–70° S and 25–32 km, near the maximum NO₂ increases. Between 20–32 km, the source of air mass was determined to be the upper stratosphere and lower mesosphere at latitudes poleward of 65–70° S and at a time during the polar night (early to mid June). For both altitude

regions, most of the transport to the region of interest occurs during the polar night. At the location and time of the O₃ minimum (in September and October at 75–90° S and at 16–25 km) the advective rates of descent and latitudinal motion were found to be very small (0.06 km per day and 1° latitude per month, respectively). This permitted a simplification in that fixed parcel calculations could be used. (As noted earlier, the advective descent rates at earlier times and higher altitudes is significantly larger.) We retain the correct time variation of temperature, pressure, sun angle and climatological overhead opacity. Because the O₃ hole occurs before the onset of the final warming^{29,32–34}, transport by transients is assumed to be relatively unimportant for the stable southern polar vortex. This is confirmed by the examination of the latitudinal transport of aerosols (as determined from satellite data) during these months (M. P. McCormick, personal communication).

Calculations were carried out at 75° S first with homogeneous chemistry only and then with both homogeneous and heterogeneous reactions included. The heterogeneous reactions referred to are reactions (10) and (11).



These reactions are known to occur on surfaces in laboratory tests³⁵, and it has been speculated that the presence of surfaces due to stratospheric aerosols and polar stratospheric clouds^{36,37} within the south polar vortex may lead to the initiation of these reactions providing higher levels of reactive chlorine (HOCl and Cl₂) than would otherwise occur, subsequently prompting the catalytic destruction of O₃ by reactions (3) and (4).

Calculations were carried out at 3-km levels between (and including) 16 and 31 km. Results of the O₃ time history for some of these levels, and the time variation of the O₃ column sum between 16 and 31 km are shown in Fig. 4. Initial conditions for the calculations were chosen to be in accord with the relative distributions of inferred species⁶ and the data discussed herein. The results show a drop in the O₃ concentrations beginning with the first sunlight at the upper levels and later at the lower levels. Without heterogeneous effects, the column sum of O₃ is reduced by 35–40% by day 290 to 295 (the third week in October). These results are in excellent agreement with the magnitude of the observations of the column O₃ decreases at the Halley Bay latitudes (75.5° S)^{29,32}, with the observed rate of O₃ column decrease³⁴, and with the satellite observations of the altitude distributions of the O₃ loss³². We note that unpublished analyses of the SAGE II and SBUV data (M. P. McCormick, S. Chandra, personal communications) indicate significant reductions of O₃ within the vortex, during the formation of the O₃ minima, up to altitudes of 45 km. This agrees with what would be expected from the proposed mechanism. Finally, the calculated growth of the NO₂ column sum, between 20 and 31 km, is within 12% of column NO₂ observations¹⁰ made at 75.5° S in September/October 1982. This provides observational evidence of significant levels of odd nitrogen present at these high latitudes during the formation of the O₃ minimum.

The chemical mechanism responsible for the O₃ decreases is the catalytic destruction of O₃ by NOY through reactions (1) and (2). After the polar night, the large mixing ratios of N₂O₅ which develop during the descending motion within the polar night vortex are photodissociated ultimately forming high levels of NO and NO₂. The chlorine cycle is ineffective due to the formation of HCl by Cl (formed primarily from the photodissociation of ClNO₃) reacting with relative high levels of CH₄ and due to reactions (5) and (6). The reactive chlorine is effectively sequestered as HCl and declining ClNO₃. Repeating calculations with chlorine levels doubled has little effect.

It is not necessary to invoke heterogeneous chemistry to explain the O₃ minimum. Nonetheless, in light of the intense interest in the atmospheric chlorine issue, we examine this effect.

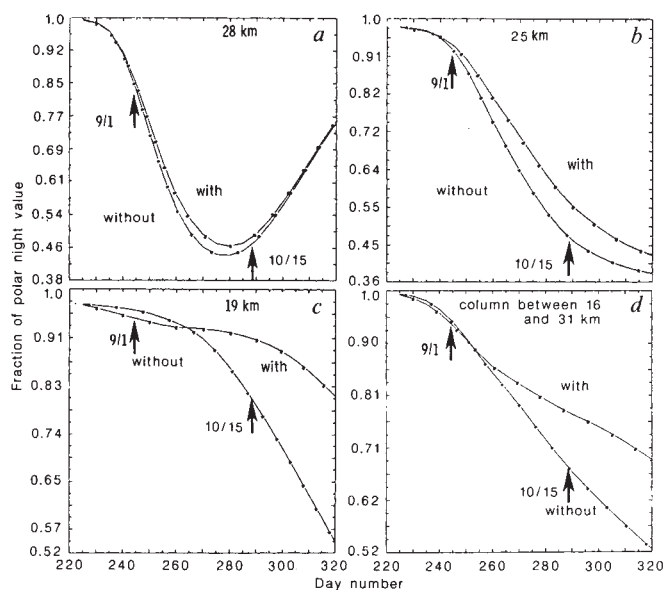
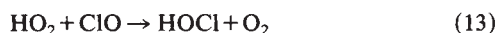
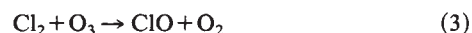
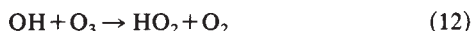


Fig. 4 *a-c*, Time history of O_3 concentration variations at indicated altitudes and $75^\circ S$. Results are shown as fractions of the last polar night values for cases run with and without heterogeneous reactions included. Initial O_3 concentrations for these calculations are 1.30×10^{12} , 2.15×10^{12} and 4.50×10^{12} molecules cm^{-3} for panels *a-c*, respectively. Heterogeneous processes were included as 'effective homogeneous reactions' in the photochemical code. Rate constants used were $10^{-16} cm^3 mol^{-1} s^{-1}$ for reaction (10) and $10^{-18} cm^3 mol^{-1} s^{-1}$ for reaction (11). These rate constants are 2-3 orders of magnitude larger than the corresponding values for purely homogeneous reactions. These rates give a photochemical lifetime for $ClNO_3$ against loss by reaction (11), the dominant reaction, of only 1.6 days. The values used are thought to represent significant overestimates of these parameters based upon the available literature. 1 September and 15 October are indicated by arrows. *d*, Time history of O_3 column density with and without heterogeneous effects between, and including, 16 and 31 km. Initial O_3 column values at 16-31 km are 4.20×10^{18} molecules cm^{-2} . Rate of column O_3 destruction observed was 0.60% per day. Calculated without heterogeneous effects, 0.66%; with, 0.50%.

The inclusion of the heterogeneous reactions (10 and 11) as "effective homogeneous" reactions with very rapid rates (see Fig. 4 legend) works to inhibit the catalytic destruction by NOY above 20 km. This is due to the formation of $ClNO_3$ reducing the amount of highly reactive NOY available and to reaction (6) which impedes the NOY catalytic cycle. Below 20 km, the heterogeneous chemistry accelerates the initial rate of destruction of O_3 due to the formation of $HOCl$ from reaction (11) with its subsequent photodissociation to form OH and Cl. The following cycle occurs:



This O_3 destruction cycle is initially more rapid than the NOY cycle or the odd chlorine cycle and persists that way for nearly 30 days at 19 km and longer at 16 km (not shown). The decline in total O_3 between 16 and 31 km will continue as long as the polar vortex remains intact. With the occurrence of the final warming (the dynamical seasonal transition), the cycle ends with the transport of the odd nitrogen to lower latitudes and O_3 to the higher latitudes from the lower latitudes. This is how the recovery of O_3 levels within the hole is accomplished.

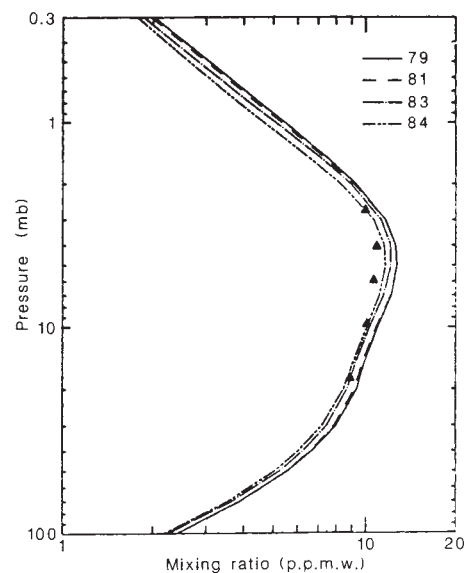


Fig. 5 Vertical profiles of zonally averaged O_3 mass mixing ratios are shown for the month of October 1979-84 for $60^\circ S$ as determined from SBUV/TOMS measurements made from the Nimbus 7 spacecraft³². Also shown on the figure are model derived values (solid triangles) showing the reductions in O_3 mass mixing ratios (referenced to the 1979 SBUV/TOMS values, solid wave) due to a 60% increase in stratospheric NOY. This NOY increase is consistent with NO_2 increases observed by the LIMS, SAGE, SME, and SAGE II data between 1979 and 1984 which are discussed in this paper. The model calculations were carried out for $60^\circ S$ and late October. The techniques used to make the photochemical calculations have been described³⁰. They were carried out with no transport and with full diurnal variations. The climatologically appropriate temperatures, pressures, seasonal overhead opacity and number of daylight hours were used. Model calculations were run until near-cyclical diurnal variations occurred (10 days or less for these altitudes and latitudes in mid October). (Satellite derived O_3 profiles after ref. 32).

Finally, we note that any proposed explanation for the occurrence of the springtime O_3 minima in Antarctica must account for its diminished presence in the Northern Hemisphere. The Northern Hemisphere winters experience significantly higher levels of transient planetary wave activity than the Southern Hemisphere. Studies of the irreversible mixing of potential vorticity associated with strong transients²⁴⁻²⁶, suggest that odd nitrogen will be transported away from the polar regions during such transients. Transport due to these transients and due to the presence of significant quasi-stationary planetary waves, always present in the northern hemisphere winter, will preclude the formation of prominent O_3 minima such as those observed in the Southern Hemisphere.

Finally, an explanation for the occurrence of the springtime O_3 minima must also account for its apparent beginnings in the mid 1970s and its persistence to the mid-1980s. The proposed relationship to the solar cycle (with the last minimum in late 1975) and the related buildup of stratospheric NOY lasting until 1984-85 is compatible with the pertinent observations. Precedent for the presence of the currently observed O_3 minima may be established by a monthly average of total O_3 of 184 Dobson units measured at Dumont D'Urville station in Antarctica ($66.7^\circ S/140^\circ E$) in September 1958³⁸. This was shortly after the maximum of the very active solar cycle 19. We note, however, that there is considerable uncertainty associated with these measurements. A more complete discussion of these observations, the heterogeneous rates (including that of N_2O_5 and H_2O), the effect of the role of transport associated with the final

warming and when it occurs, the effect of transients before the final warming, and sensitivities to latitude and altitude is beyond the scope of this paper. These issues are treated elsewhere¹¹. The results of that paper offer further support for the proposed mechanism and the conclusions drawn herein.

Mid-latitude ozone reductions from 1979 to 1984. Total ozone over North America, Europe and Asia has recently been reported³⁹ as having reached the lowest levels since 1958 (the time of the last large solar maximum). Data used in this analysis were from satellite and ground-based instruments and show a 5–7% decrease by 1982 compared to long-term seasonal means.

Data from the SBUV/TOMS experiment aboard the Nimbus 7 spacecraft have also been examined^{32,40} and show a substantial reduction in stratospheric O₃ between 1979 and 1984. Reductions in the October monthly means of approximately 10–15% were observed from 23 km to the stratopause at 60° S. Reductions at other latitudes were also observed.

We suggest that the O₃ reductions that have been reported are due in large part to catalytic destruction by increasing NOY through reactions (1) and (2). This proposition is evaluated in a preliminary way with the aid of diurnal calculations carried out for late October at five altitudes at 60° S. We have made

two sets of calculations, one using NOY levels as derived from the October 1978 LIMS measurements⁶. The other calculation is for similar conditions but uses NOY derived from the NO₂ levels measured in 1982 and 1984 by the SME and the SAGE II experiments. Shown in Fig. 5, along with the measured O₃ reductions, are the calculated reductions in O₃ (compared to the 1979 values) associated with increases of NOY of 60%. These NOY increases are consistent with the previously discussed increases in observed NO₂ and inferred NOY between 1979 and 1984. The predicted O₃ decreases agree very well with the observed O₃ decreases suggesting that the observed and reported changes in O₃ during the 1979–84 period may, in fact, be due to the large infusions of NOY that have occurred during solar cycle 21. Changes in O₃ at these lower latitudes (where the NO₂ increases are largest) are clearly not as large as those which cause the presence of the high-latitude O₃ hole. This is principally due to the larger rate of O₃ production present near 50–60° S and at altitudes of 30–35 km. This higher production rate is due to the reduced overhead opacity (a factor of 6 less) at these lower southern latitudes and higher altitudes.

We thank Dr M. P. McCormick for preliminary SAGE II NO₂ data and Dr P. K. Bhartia for providing SBUV/TOMS data.

Received 31 March; accepted 10 September 1986.

- Crutzen, P. J. *Q. J. R. Met. Soc.* **96**, 320 (1970).
- Russell, J. M. III *et al. J. geophys. Res.* **89**, 5099–5108 (1984).
- Chu, W. P. & M. P. McCormick, *J. geophys. Res.* **91**, 5465–5476 (1986).
- Mount, G. H. *et al. Geophys. Res. Lett.* **10**, 265–268 (1983).
- Mount, G. H., Rusch, D. W., Noxon, J. F., Zawodny, J. M. & Barth, C. A. *J. geophys. Res.* **89**, 1327–1340 (1984).
- Callis, L. B., Natarajan, M., Boughner, R. E., Russell, J. M. III & Lambeth, J. D. *J. geophys. Res.* **91**, 1167–1199 (1986).
- Ko, M. K. W., Tung, K. K., Weinstein, D. K. & Sze, N. D. *J. geophys. Res.* **90**, 2312–2329 (1985).
- Isaksen, I. S. A. & Stordal, F. *J. geophys. Res.* **91**, 5249–5263 (1986).
- WMO Global Ozone Research and Monitoring Project, Report No. 11 (WMO, Geneva, 1981).
- McKenzie, R. L. & Johnston, P. V. *Geophys. Res. Lett.* **11**, 73–75 (1984).
- Callis, L. B. & Natarajan, M. *J. geophys. Res.* **91**, 10771–10796 (1986).
- Jackman, C. H., Frederick, J. E. & Stolarski, R. S. *J. geophys. Res.* **85**, 7495–7506 (1980).
- Solomon, S., Crutzen, P. J. & Roble, R. G. *J. geophys. Res.* **87**, 7206–7220 (1982).
- Ko, M. K. W., McElroy, M. B., Weinstein, D. K. & Sze, N. D. *J. geophys. Res.* **91**, 5395–5404 (1986).
- Crutzen, P. J. *A. Rev. Earth planet. Sci.* **7**, 443 (1979).
- Ströbel, D. F., Hüntel, D. M. & McElroy, M. B. *J. geophys. Res.* **75**, 4307 (1971).
- Brasseur, G. & Nicolet, M. *Planet. Space Sci.* **21**, 939 (1973).
- Garcia, R. R., Solomon, S., Roble, R. & Rusch, D. W. *Planet. Space Sci.* **32**, 411–423 (1984).
- Russell, J. M. III, Solomon, S., Gordley, L. L., Remsberg, E. E. & Callis, L. B. *J. geophys. Res.* **89**, 7267–7275 (1984).
- Horvath, J. J. & Frederick, J. E. *Geophys. Res. Lett.* **12**, 495–497 (1985).

- McPeters, R. D. in *Atmospheric Ozone* (eds Zerefos, C. S. & Ghazi, A.) 184–187 (Reidel, Dordrecht, 1985).
- Callis, L. B., Russell, J. M. III, Natarajan, M. & Haggard, K. V. *Geophys. Res. Lett.* **10**, 945–948 (1983).
- Callis, L. B., Boughner, R. E. & Lambeth, J. D. *J. geophys. Res.* (in the press).
- McIntyre, M. P. & Palmer, T. N. *Nature* **305**, 593–600 (1983).
- Dunkerton, T. & Delisi, D. P. *J. geophys. Res.* **91**, 1199–1208 (1986).
- Butchart, N. & Remsberg, E. E. *J. Atmos. Sci.* (in the press).
- Rusch, D. W. & Sharp, W. E. *J. geophys. Res.* **86**, 100–111 (1981).
- Solar Geophysical Data Prompt Reports, National Oceanic and Atmospheric Administration* No. 495 (1) (1985).
- Farman, J. C., Gardiner, B. G. & Shanklin, J. D. *Nature* **315**, 207–210 (1985).
- Natarajan, M., Callis, L. B., Boughner, R. E., Russell, J. M. III & Lambeth, J. D. *J. geophys. Res.* **91**, 1153–1166 (1986).
- JPL Publ. 83-57 (NASA Jet Propulsion Laboratory, Pasadena, 1985).
- Bhartia, P. K., Heath, D. F. & Fleig, A. F. *Symp. Dynamics and Remote Sensing of the Middle Atmosphere, 5th Scientific Assembly of IAGA* (Prague, Czechoslovakia, 5–17 August, 1985).
- Krueger, A. J., Stolarski, R. S., Alpert, J. C., Heath, D. F. & Chandra, S. *EOS* **66**, 838 (1985).
- Stolarski, R. S. *et al. Nature* **322**, 808 (1986).
- Molina, L. T., Molina, M. J. & Tom, R. D. *J. phys. Chem.* **89**, 3779–3781 (1985).
- McCormick, M. P., Steele, H. M., Hamill, P., Chu, W. P. & Swisler, T. J. *J. Atmos. Sci.* **39**, 1387–1397 (1982).
- Hofman, D., Rosen, J. M. & Gringel, W. *J. geophys. Res.* **90**, 2341–2354 (1985).
- Repapis, C. C., Zerefos, C. S., Jenne, R. L. & Kotini, S. *Twenty Years of Total Ozone Observations for the World* (Academy of Athens, Research Centre for Atmospheric Physics and Climatology, Publ. No. 2, Athens, 1980).
- Angell, J. K., Korshover, J. & Planet, W. G. *Mon. Weath. Rev.* **113**, 641–646 (1985).
- Heath, D. F., Bhartia, P. K. & Schlesinger, B. M. *EOS* **66**, 1009 (1985).

Compensatory mutations suggest that base-pairing with a small nuclear RNA is required to form the 3' end of H3 messenger RNA

Fred Schaufele, Gregory M. Gilmartin, Willi Bannwarth* & Max L. Birnstiel

Institut für Molekularbiologie II der Universität Zürich, CH-8093 Höggerberg, Zürich, Switzerland

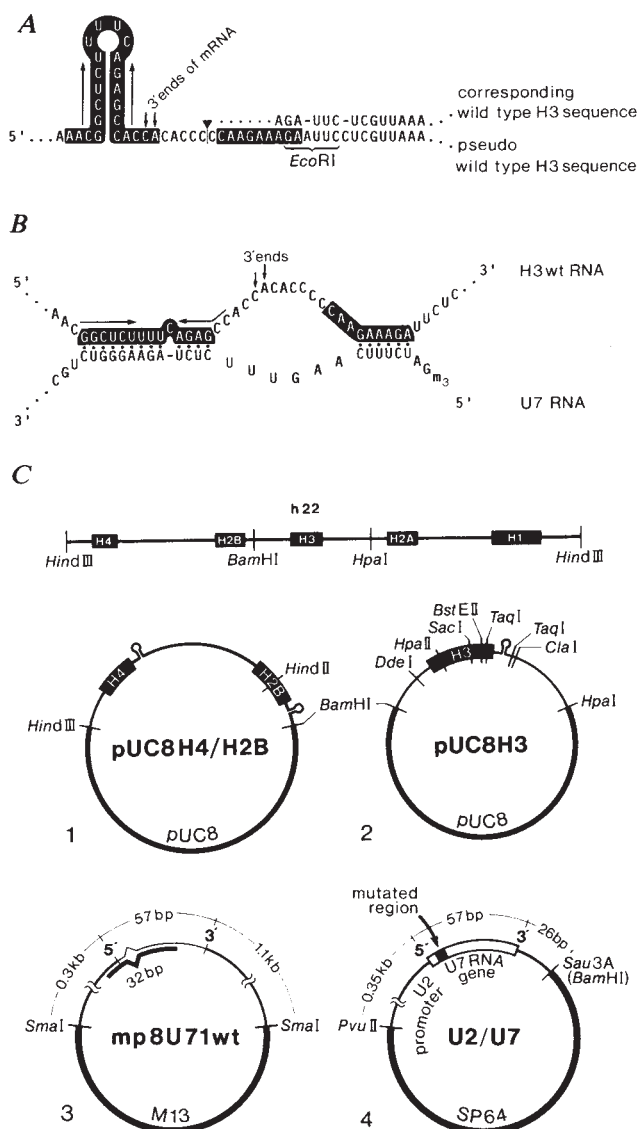
* Central Research Units, Hoffmann-La Roche, CH-4002, Basle, Switzerland

Processing of the 3' end of sea urchin H3 histone pre-mRNA requires conserved sequence elements and the presence of U7 snRNA. A mutation in the conserved CAAGAAAGA sequence of the H3 pre-mRNA that renders 3' processing of this precursor defective is shown to be suppressed by a compensatory change in the U7 snRNA, restoring the base-pairing potential of the two RNAs. RNA-RNA contacts between these two molecules appear to be an essential feature of the 3' processing reaction.

THE copying of a gene into its RNA product is a complex process involving the initiation of the RNA polymerase at the transcription start site followed by elongation of the transcript past the point which will become the 3' end of the mature

message. The extended RNA thus produced is edited by one or several RNA cleavage steps. One such cleavage step yields the 3' end of the mRNA in a process known as RNA 3' processing (reviewed in ref. 1). Internal RNA segments (introns) are also

Fig. 1 **A**, Nucleotide sequence of the sea urchin histone embryonic H3 precursor RNA spanning the 3' processing cleavage sites ($\downarrow$). Highly conserved sea urchin histone sequence elements are shown white on black. **B**, Hypothetical base-pairing model between the U7 snRNA and the wild-type H3 histone precursor RNA¹⁴. Dots, potential contacts of the U7 snRNA with the highly conserved histone sequences. **C**, plasmids used in microinjection studies: (1) pU-H3wt, a 1,600 bp BamHI to HpaI insert spanning the H3 gene of the sea urchin, *Psammechinus miliaris*, h22 early histone repeat unit⁴⁰ into BamHI/HindIII digested pUC8 in which the polylinker EcoRI site had been destroyed; pU-H3 mutants are identical to this except for indicated base changes (Fig. 2C). (2) pU-H4/H2B, a 2,366 HindIII to BamHI insert spanning the H4 and H2B genes of the same cluster as pU-H3wt. (3) mp8-U71wt, starting clone for U7 gene mutagenesis contains an approximately 1,500 bp AluI to EcoRI fragment including the U71 gene⁴¹ inserted into the SmaI site of M13mp8 such that the RNA-like strand is incorporated into the phage. (4) U2/U7, expression vector for the production of the U7 RNA sequences in the *Xenopus* oocyte (modified from ref. 18). **Methods.** The EcoRI site downstream of the CAAGAAAGA element was introduced²⁶ by the linker-scanning procedure. This sequence was recombined into another vector containing a unique SmaI site far downstream of the unique EcoRI site. The construct was restricted with EcoRI, resected with Bal31 nuclease (to the sequence, CCC $\uparrow$ in Fig. 1A), then digested with SmaI and reclosed, thereby reconstructing a new SmaI site (CCC $\uparrow$ GGG). Permutations of the oligonucleotides described in the text could be cloned between this reconstructed SmaI site and the EcoRI linker (representing the 5' and 3' arms of the H3 gene, respectively) in order to generate the H3 mutants. Mutant construction was confirmed by sequencing. The U7CAGA3sup mutant was constructed by M13 mutagenesis³². A 32-nucleotide-long oligonucleotide, complementary to the mp8-U71wt phage DNA except for two nucleotides, UC where the clone has CU (=◇= in Fig. 1C, 3) was made; 15 bp complementarities were present on either side of this mismatch. After hybridization of this oligonucleotide and the M13 universal primer to mp8-U71wt, extending with Klenow polymerase and ligating, the recombinants were grown on JM103 and individual plaques analysed by hybridization with the radio-labelled 32-mer oligonucleotide under increasing stringency. Those plaques which still gave a signal when control mp8-U71wt plaques did not were analysed for the presence of a newly constructed Hinfl site (GATTC, see Fig. 3B and confirmed by sequencing. bp, base-pairs.



removed from almost all pre-mRNAs in a reaction referred to as RNA splicing (see refs 2-6 for reviews).

RNA splicing of nuclear pre-mRNAs⁷⁻¹⁰, the maturation of transfer RNA transcripts^{11,12}, and the 3' processing of histone pre-mRNAs¹³⁻¹⁵ and of the precursors to polyadenylated RNAs^{16,17} all require the presence of trans-acting nuclear RNPs. The U7 snRNP is the small RNP species that supports histone 3' processing^{13,14,18} and the U1 snRNP is known to participate in intron excision during splicing of polyadenylated precursor RNAs⁷⁻¹⁰. Both the U7 (ref. 14) and the U1 (refs 19, 20) snRNA species have the potential to base-pair with the precursor RNA near the cleavage site, which suggests that the precursor and these snRNAs form RNA-RNA hybrids as intermediates of the RNA cleavage reaction.

All known cell cycle-regulated histone mRNAs of all species, except yeast, have a virtually identical palindrome near the 3' end of the mature mRNA^{1,21}, which is followed a few nucleotides downstream by a conserved spacer sequence^{1,22}. The spacer sequence is highly conserved across sea urchin histone genes as a nine nucleotide block, CAAGAAAGA²³. The potential base-pairing interactions with sea urchin U7 snRNA¹⁴ (see Fig. 1B) lie completely within these two highly conserved sea urchin histone sequence elements (Fig. 1A). Both the palindromic and the downstream sequences have been demonstrated to be essential for 3' processing of sea urchin histone precursor RNAs²⁴⁻²⁶. Analogous marine histone precursor RNA sequences have also

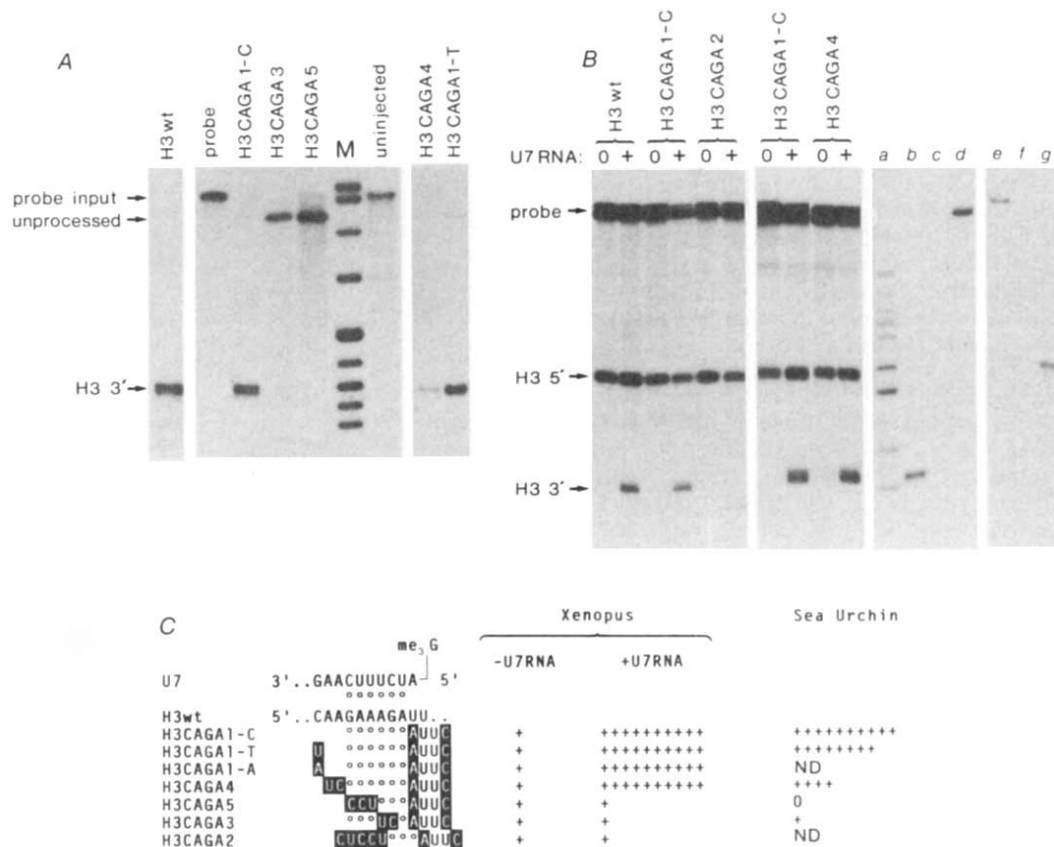
been shown to be necessary for 3' processing²⁷, via an endonucleolytic cleavage¹⁵, as well as for cell cycle regulation²⁷.

Here we examine how these base complementarities are relevant to 3' processing. In particular, we have investigated those interactions predicted to occur downstream of the cleavage site(s), within the CAAGAAAGA element. We first define which downstream nucleotides are essential for 3' processing in sea urchin embryos and for U7 snRNA-dependent processing in the *Xenopus* oocyte, using mutagenesis of the histone H3 gene. We then show that a processing-deficient histone H3 mutant can be rescued by coinjection into *Xenopus* oocytes with a U7 gene in which Watson-Crick-type base complementarities to the mutant histone pre-mRNA are restored. The GAAAGA sequence within the histone pre-mRNA is thus a target for RNA-RNA duplex formation with the 5' terminal sequence of the U7 snRNA.

Mutagenesis

A series of mutants in the H3 CAAGAAAGA sequence element was constructed in which single or small groups of bases were altered. The wild-type distance between the terminal palindrome and the CAAGAAAGA element was maintained in all mutants since this is known to be a critical requirement for efficient 3' processing of the histone precursor RNA²⁶. Mutant construction is detailed in Fig. 1A. Briefly, a cloned H3 gene of the sea urchin, *Psammechinus miliaris*, containing an EcoRI linker inserted

Fig. 2 A, SP6 mappings of sea urchin blastulae (6.25 h post-fertilization) developed after injection with $\sim 10^4$ molecules each of control pU-H4/H2B and test pU-H3 mutant DNAs, *Bam*HI linearized. M, pBR322 *Hpa* II digested size markers. The H3 probe extends from *Sac*I to *Cl*aI (Fig. 1C, 2); the H2B probe is from *Hind*III to *Bam*HI (Fig. 1C, 1). B, *S*₁ mappings of *Xenopus* oocytes incubated 16–18 hours after injection with $\sim 2 \times 10^8$ molecules of circular pU-H3 mutant DNAs. Oocytes have either been preinjected (+) or not (0) with $\sim 1 \times 10^6$ molecules of U7 snRNA lanes c, f uninjected (control mappings of uninjected *Xenopus* oocytes); lane a pBR322 *Hpa*II digested size markers: lanes b, g, sea urchin RNA (control map with *Psammechinus* 32-cell stage RNA); lane d, 3' probe (from *Bst*EII to *Cl*aI, Fig. 1C, 2); lane e, 5' probe (from *Hpa*II to *Dde*I, Fig. 1C, 2). C, Schematic representation of the pU-H3 histone mutants and their ability to be processed in sea urchins or *Xenopus*. O, base-pairing potentials with the conserved CAAGAAAGA sequence; nucleotides are mutated from the H3wt sequence to that indicated (white on black); +, qualitative representation of the ability of a given mutant to be processed; ND, not determined; me3G, trimethyl cap structure at 5' end of the U7 snRNA¹⁸.



immediately downstream of the CAAGAAAGA element (Fig. 1A; ref. 26), was restricted with *Eco*RI and then resected by *Bal*31 digestion to the sequence, CCC (T in Fig. 1A), immediately upstream of the CAAGAAAGA element. The sequences between the CCC and *Eco*RI sites were then replaced by the synthetic complementary DNA sequences 5'-CCAAGAAAG-3' and 3'-GGTCTTTCTTAA-5' or permutations of these sequences, generating a series of defined mutations in this region. Because of the cloning strategy, all mutations share the insertion of an extra T and C downstream of the conserved CAAGAAAGA sequence (see Fig. 1A) but these sequence alterations proved to have no effect on 3' processing. Processing of this 'pseudowild-type' mutant (H3CAGA1-C) was 104% as efficient as wild-type histone pre-mRNA (averaged over seven *Xenopus* oocyte injection experiments). Similarly, the pseudowild-type mutant was processed in sea urchin embryos as efficiently as wild-type H3 RNA (data discussed below).

H3 mutants in sea urchin embryos

The effect of these mutations on 3' processing was tested by microinjection into sea urchin embryos (refs 28, 29 and L. Vitelli, I. Kemmler, M. Busslinger and M.L.B., unpublished data) and into *Xenopus* oocytes³⁰. When sea urchin 'embryonic' histone genes, including the H3 gene, are microinjected into sea urchin eggs, they are transcribed after fertilization in the same developmentally regulated fashion as the endogenous embryonic histone genes (Vitelli *et al.*, unpublished data). To distinguish between RNAs transcribed from the microinjected *Psammechinus* histone constructs and the endogenous histone genes, the cloned genes were microinjected into eggs of the sea urchin species, *Paracentrotus lividus*. Histone RNAs from this species are sufficiently

divergent from *Psammechinus* transcripts that they do not interfere in RNA mapping experiments.

Sea urchin eggs were injected with linearized wild-type or mutant pU-H3 constructs together with a pU-H4/H2B plasmid (see Fig. 1C) which served as the injection control. The manipulated eggs were fertilized and allowed to develop to early blastulae, a point at which the accumulation of embryonic histone mRNA reaches a maximum³¹. The generation of H3 mRNAs with correct 3' ends was analysed by SP6 mapping (Fig. 2A); 3' ends of transcripts from the coinjected *Psammechinus* embryonic histone H2B gene were similarly mapped to compare the efficiency of the injections. Production of H2B transcripts from all injections within a series was essentially the same (data not shown), but the ability of the various H3 constructs to produce RNAs with correct 3' ends (H3 3' in Fig. 2A) varied considerably. A strong band corresponding to RNase protected long transcripts which extended through to the 3' end of the probe homology ('unprocessed' RNA in Fig. 2A) was observed in many mutants and appeared to be inversely correlated with the ability to generate correct 3' ends. A description of the tested H3 mutants and a qualitative depiction of their susceptibility to processing is presented in Fig. 2C (discussed below).

H3 mutants in *Xenopus* oocytes

The H3 mutants were also analysed by injection into *Xenopus* oocytes. In this heterologous system the efficient production of sea urchin H3 RNA termini is dependent upon coinjection into the *Xenopus* oocyte of either homologous sea urchin U7 snRNA¹⁴ or vectors expressing that U7 snRNA¹⁸. The *Xenopus* U7 snRNA seems to be too divergent to be able to serve the sea urchin H3 gene, which therefore requires the sea urchin U7 snRNA for efficient 3' processing. To assess the efficiency of

processing in the various H3 mutants, both 5' and 3' ends of the H3 transcripts generated in the oocytes were quantified by S_1 mapping (Fig. 2B).

When the H3 wild-type and CAAGAAAGA mutant genes were injected into the *Xenopus* oocyte nucleus in the absence of added U7 snRNA, none of the constructs yielded transcripts with the correct 3' end. In the presence of injected U7 snRNA the mutants varied considerably in their ability to form 3' ends but the wild-type H3 gene produced correct 3' ends in high yield (H3 3' in Fig. 2B). The number of H3 3' ends produced relative to the number of correctly initiated H3 transcripts (H3 5' in Fig. 2B) is the criterion used to judge the efficiency of 3' end formation, summarized in Fig. 2C.

Consensus sequence and 3' maturation

In both sea urchin and *Xenopus* oocyte microinjection experiments, the 'pseudowild-type' H3 RNA produced from the H3CAGA1-C mutant (see Fig. 2C) was as efficient in producing correct 3' ends as the wild-type H3 (H3wt) RNA (Fig. 2A,B). The mutations were compared with the pseudowild-type (H3CAGA1-C) gene. Mutation of the first C nucleotide of the conserved CAAGAAAGA sequence (Fig. 2C, H3CAGA1-A, H3CAGA1-T) or the following AA dinucleotide (H3CAGA4) had no measurable effect on the processing of these mutants in *Xenopus* oocytes. However, an H3 mutant in which the CAA nucleotides and the five contiguous nonconserved nucleotides immediately upstream were replaced by an *EcoRI* linker has been shown to be deficient in 3' processing in *Xenopus* oocytes²⁶. It is thus possible that simultaneous alteration of all three nucleotides, CAA, may inhibit 3' processing. We note that the H3CAGA1-T mutant was mildly defective when tested in the sea urchin system (Fig. 2A) and the H3CAGA4 construct was somewhat more defective. Hence, this sequence, CAA, conserved only in sea urchins, contributes to efficient histone RNA maturation in sea urchins. All mutations made within the GAAAGA portion of the CAAGAAAGA element, that is, inside the proposed region of U7 snRNA complementarity (H3CAGA2, H3CAGA3, H3CAGA5) definitely decreased 3' processing by a factor of at least twenty in both test systems (Figs 2A,B, 3A). Thus the mutagenesis results were consistent with the model shown in Fig. 1B: mutations which disrupt the proposed base-pairing between the U7 and H3 RNAs very strongly affect U7 snRNA-dependent processing in *Xenopus* oocytes and 3' end formation in sea urchins. It is interesting to note that the GAAAGA sequence, complementary to U7 snRNA and here identified as an essential sequence element, coincides with the 'core' sequence Pu AAAGA^{1,22} which is present in a similar position with respect to the conserved palindromic sequence in a number of vertebrate histone genes.

Requirement for base-pairing

If base complementarities between the GAAAGA sequence and the 5' portion of the U7 snRNA are important in 3' processing, then a modified U7 snRNA which has the potential to base-pair with a processing-defective H3 pre-mRNA mutant, such as the mutant H3CAGA3 should allow correct 3' end formation. To test this hypothesis, we used the genetic complementation approach in the *Xenopus* oocyte¹⁸ in which, in contrast to the sea urchin system, it is easy to show that H3 pre-mRNA processing depends on added U7 snRNA. In this complementation assay H3 transcripts are produced from the injected sea urchin histone gene. A functional sea urchin U7 snRNA is, in turn, generated in the *Xenopus* oocyte under the control of a *Xenopus* U2 promoter¹⁸ (Fig. 1C, U2/U7). Injection of wild-type histone H3 DNA (H3wt) into the *Xenopus* oocyte yielded few, if any, correct 3' ends unless coinjected with a wild-type U7 gene construct (Fig. 3A, lane 1). As anticipated from the above experiments, injection of the processing deficient H3CAGA3 mutant into the *Xenopus* oocyte nucleus yielded no correct 3' ends, even when complemented with a gene construct coding

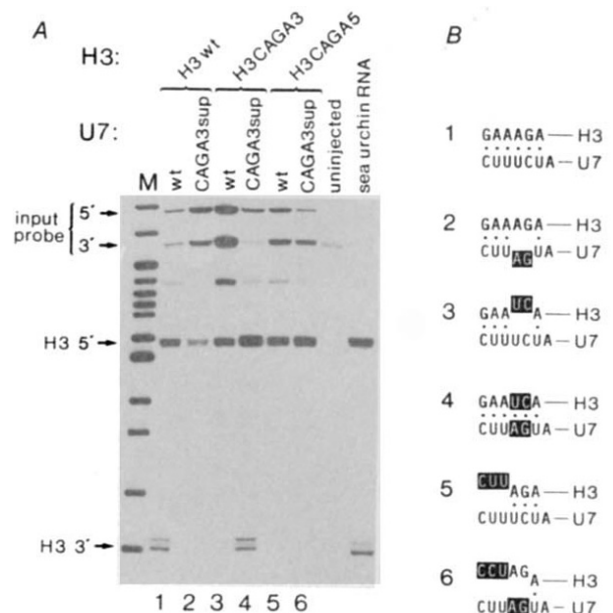


Fig. 3 A, Genetic complementation of histone processing¹⁸ with U2/U7 constructs in the *Xenopus* oocyte. The apparent heterogeneity of the 3' ends, spaced two nucleotides apart, is accentuated by the use of a 3' probe (*TaqI* to *TaqI*, Fig. 1C, 2) shorter than that used in Fig. 2B. The 5' probe is as in Fig. 2B. B, Schematic representation of U7 and H3 mutant combinations tested in Fig. 3A. Mutated nucleotides are indicated white on black. Dots, potential base-pairs between U7 and H3 RNAs.

for wild-type U7 (U7wt) snRNA sequences (Fig. 3A, lane 3). We then examined whether 3' processing activity of this mutant could be regained by introducing a complementary sequence change within the U7 snRNA. The appropriate U7 mutant, containing the nucleotide changes CU to GA (termed U7CAGA3sup, the suppressor of H3CAGA3 mutant; see Fig. 3B) was constructed by M13 mutagenesis³² (Fig. 1 legend). When expressed in *Xenopus* oocytes under the control of the *Xenopus* U2 promoter, U7CAGA3sup sequences rescued the production of 3' ends of transcripts from the H3CAGA3 histone mutant (Fig. 3A, lane 4); the 3' ends detected by S_1 mapping were identical to those observed for authentic sea urchin RNA (compare lane 4 to 'sea urchin RNA'). In contrast, when U7CAGA3sup was coinjected with either the wild-type H3 gene unit (lane 2) or another processing-defective histone mutant (H3CAGA5, lane 6) no mature histone 3' ends were produced. Thus, correct histone 3' ends were only produced when a histone RNA and a U7 snRNA were present which could base-pair with each other at the positions indicated in black in Fig. 3B (U71wt + H3wt; U7CAGA3sup + H3CAGA3).

RNA-RNA hybrids as processing intermediates

RNA base-pairing is a recurrent theme of RNA processing reactions. The mechanistic importance of complementary RNA sequences has been most clearly demonstrated where complementary 'second site' mutations in RNA can be tested directly *in vitro* in the absence of proteins as is possible for those introns which carry within themselves the information for their own removal^{33,34}. But even in *in vivo* test systems, compensatory base changes in RNA have provided an extremely useful, although indirect, analytical tool. The structure of tRNA³⁵, the molecular mechanisms of suppression in protein synthesis (ref. 36 and refs therein), class I intron excision^{37,38}, the importance of the terminal palindromes of histone mRNA to 3' maturation²⁴ and the interaction between U1 and snRNA and the exon/intron 5' splice junctions of the adenovirus E1A pre-mRNA³⁹ have all been studied in this way. This general approach was applied to

the study of histone pre-mRNA 3' processing after we noted that the U7CAGA3sup snRNA described above was capable of being precipitated with anti-Sm antibodies (G.M.G. unpublished results) and, on this basis, appears to be indistinguishable from wild-type U7 snRNA with regard to RNP formation.

This study considerably strengthens the hypothesis of essential RNA-RNA contacts during 3' processing between the downstream conserved GAAAGA box of sea urchin histone pre-mRNA and the 5' terminal sequences of the U7 snRNA¹⁴. These may be used to position the processing complex, exploiting the

high efficiency and accuracy of complementary RNA-RNA duplexes.

We thank Fritz Ochsenbein for preparing the figures, Christopher Southgate for the H2B mapping clone, M. Chipchase for reading the manuscript and Klaus Kalusa for synthesizing oligonucleotides for us. This work was supported by the Kanton Zürich and the Swiss National Science Foundation, grant 3.484.83. F.S. was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada.

Received 1 September; accepted 24 September 1986.

1. Birnstiel, M. L., Busslinger, M. & Strub, K. *Cell* **41**, 349-359 (1985).
2. Cech, T. *Cell* **34**, 713-716 (1983).
3. Greer, C. L. & Abelson, J. *Trends biochem. Sci.* **9**, 139-141 (1984).
4. Keller, W. *Cell* **39**, 423-425 (1984).
5. Sharp, P. A. *Cell* **42**, 397-400 (1985).
6. Cech, T. R. *Cell* **44**, 207-210 (1986).
7. Padgett, R. A., Mount, S. M., Steitz, J. A. & Sharp, P. A. *Cell* **35**, 101-107 (1983).
8. Krämer, A., Keller, W., Appel, B. & Lührmann, R. *Cell* **38**, 299-307 (1984).
9. Krainer, A. R. & Maniatis, T. *Cell* **42**, 725-736 (1985).
10. Black, D. L., Chabot, B. & Steitz, J. A. *Cell* **42**, 737-750 (1985).
11. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. *Cell* **35**, 849-857 (1983).
12. Gold, H. A. & Altman, S. *Cell* **44**, 243-249 (1986).
13. Galli, G., Hofstetter, H., Stunnenberg, H. G. & Birnstiel, M. L. *Cell* **34**, 823-828 (1983).
14. Strub, K., Galli, G., Busslinger, M. & Birnstiel, M. L. *EMBO J.* **3**, 2801-2807 (1984).
15. Gick, O., Krämer, A., Keller, W. & Birnstiel, M. L. *EMBO J.* **5**, 1319-1326 (1986).
16. Moore, C. L. & Sharp, P. A. *Cell* **36**, 581-591 (1984).
17. Moore, C. L. & Sharp, P. A. *Cell* **41**, 845-855 (1985).
18. Strub, K. & Birnstiel, M. L. *EMBO J.* **5**, 1675-1682 (1986).
19. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. *Nature* **283**, 220-224 (1980).
20. Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1877-1879 (1980).
21. Wells, D. E., *Nucleic Acids Res.* **14**, r119-r149 (1986).

22. Turner, P. C., Aldridge, T. C., Woodland, H. R. & Old, R. W. *Nucleic Acids Res.* **11**, 4093-4107 (1983).
23. Hentschel, C. C. & Birnstiel, M. L. *Cell* **25**, 301-313 (1981).
24. Birchmeier, C., Folk, W. & Birnstiel, M. L. *Cell* **35**, 433-440 (1983).
25. Birchmeier, C., Schümperli, D., Sconzo, G. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1057-1061 (1984).
26. Georgiev, O. & Birnstiel, M. L. *EMBO J.* **4**, 481-489 (1986).
27. Stauber, C., Lüscher, B., Eckner, R., Lötscher, E. & Schümperli, D. *EMBO J.* (in the press).
28. McMahon, A. P. *et al. Devl Biol.* **108**, 420-430 (1985).
29. Flytzanis, C. N. *et al. Devl Biol.* **108**, 431-442 (1985).
30. Probst, E., Kressman, A. & Birnstiel, M. L. *J. Molec. Biol.* **135**, 709-732 (1979).
31. Maxson, R. E. & Wilt, F. H. *Devl Biol.* **94**, 435-440 (1982).
32. Zoller, M. J. & Smith, M. *Meth. Enzym.* **100**, 468-500 (1983).
33. Burke, J. M. *et al. Cell* **45**, 167-176 (1986).
34. Waring, R. B., Townner, P., Minter, S. J. & Davies, R. W. *Nature* **321**, 133-139 (1986).
35. Smith, J. D., Anderson, K., Cashmore, A., Hooper, M. L. & Russell, R. L. *Cold Spring Harb. Symp. Quant. Biol.* **35**, 21-27 (1970).
36. Normanly, J., Ogden, R. C., Horvath, S. J. & Abelson, J. *Nature* **321**, 213-219 (1986).
37. Weiss-Brummer, B., Holl, J., Schweyen, R. J., Rödel, G. & Kaudewitz, F. *Cell* **33**, 195-202 (1983).
38. Höll, J., Rödel, G. & Schweyen, R. J. *EMBO J.* **4**, 2081-2085 (1985).
39. Zhuang, Y. & Weiner, A. M. *Cell* **46**, 827-835 (1986).
40. Clarkson, S. G., Smith, H. O., Schaffner, W., Gross, K. W. & Birnstiel, M. L. *Nucleic Acids Res.* **3**, 2617-2632 (1976).
41. De Lorenzi, M., Rohrer, U. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3243-3247 (1986).

LETTERS TO NATURE

Is the Universe dominated by relativistic particles?

Ricardo A. Flores*, George R. Blumenthal†, Avishai Dekel‡ & Joel R. Primack¶

* Department of Physics, Brandeis University, Waltham, Massachusetts 02254, USA and Theory Division, CERN, 1211 Geneva 23, Switzerland

† Lick Observatory, Board of Studies in Astronomy and Astrophysics, University of California, Santa Cruz, California 95064, USA

‡ Racah Institute of Physics, The Hebrew University, Jerusalem, Israel

¶ Santa Cruz Institute for Particle Physics, University of California, Santa Cruz, California 95064, USA

The decaying dark matter (DM) cosmology¹⁻³ attempts to reconcile the theoretical prejudice that the total energy density of the Universe, $\Omega = 1$ with the observations that the matter clustered on scales up to a few megaparsecs amounts only to $\Omega_{cl} = 0.1-0.3$. Decaying DM postulates that a heavy-elementary-particle species X first drives the formation of galaxies and clusters, and then decays nonradiatively, providing a smooth, undetected background of relativistic particles that at present contribute Ω_r to the total energy density of the Universe, Ω . We consider here the effects of decaying DM on the radial distribution of mass in spiral galaxies, assumed to have formed through dissipative collapse inside a gravitationally induced protogalaxy consisting initially of a homogeneous mixture of dissipationless DM and a small fraction of dissipative baryonic material⁶. The baryonic inner parts of galaxies are self gravitating, but mass loss from X decay causes the rotation velocity in the outer parts to decrease. We find that the observed flat rotation curves cannot be obtained in these decaying DM models. Thus, a relativistic, weakly interacting decay product cannot be dominant.

In the original models¹⁻³ ('type I') the non-relativistic matter contributes $\Omega_{nr} = \Omega_{cl}$ and $\Omega_r = 1 - \Omega_{nr} \approx 0.8$. Another possibility is that the Universe becomes dominated by a primordial, stable non-relativistic DM species Y after decay of the unstable species X. In this model⁷ (type II), X decay unbinds a large fraction of the Y particles, causing them to stream away from clusters, providing a relatively smooth background of nonrelativistic DM. Our results constrain but do not exclude the type II model.

There are several astrophysical constraints on the redshift $z_d(t = \tau_d)$ corresponding to the epoch of X decay. An upper limit $1 + z_d \leq 5$ arises from the isotropy of the microwave background⁸⁻¹⁰, from the requirement that the cores of rich clusters remain in virial equilibrium¹¹, and from a decaying DM model for gravitational lenses¹². On the other hand, a lower limit $1 + z_d \geq 20$ ensures, in the linear theory, a small enough value of our infall velocity towards Virgo in type I models¹¹; however, this bound is exponentially sensitive to observational uncertainties and is affected by the nonsphericity and nonlinearity of the infall^{13,14}, so a value $1 + z_d \approx 5$ is not excluded. We assume that by $t_d \approx 10^9$ yr the inner parts of galaxies have collapsed and dissipated, as the corresponding dynamical and cooling times are $< t_d$.

The response of a protogalaxy to the dissipational collapse of its baryonic component and the decay of a fraction of its DM component can be described by an approximate analytical model⁶. For a particle in circular orbit about a spherically symmetric mass distribution $M(r)$, $rM(r)$ is an adiabatic invariant provided that $M(r)$ changes slowly with time. Because there is more phase space available for nearly circular orbits than for nearly radial orbits, we make the simplifying approximation that the orbits of DM particles are circular. Actually, $r_{max}M(r_{max})$ is also an adiabatic invariant for purely radial orbits if $M(r)$ varies in a self-similar fashion.

Assume that the initial, spherically symmetrical mass distribution of the galaxy $M_i(r)$ is in dynamical equilibrium, with a constant fraction $F_i \equiv M_b/M_i$ of baryons, where M_b is the total mass in baryons and M_i is the initial total mass. First, as the

¶ Present address: Department of Physics, The Weizmann Institute of Science, Rehovot 76100, Israel.

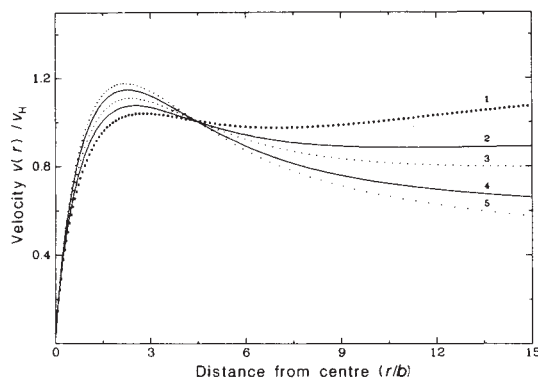


Fig. 1 Rotation curves calculated with the analytical model; $v(r)$ is the rotational velocity, in the plane of the disk, needed by a particle to remain in circular orbit at distance r/b from the centre and $v_H = v(r = R_H = 4.5b)$. $a = 0.42 R$. Curve 1: $F = 0.05$; $f = 0.75$; $b/R = 0.05$; $\lambda = 0.07$. Curve 2: $F = 0.10$; $f = 0.75$; $b/R = 0.05$; $\lambda = 0.07$. Curve 3: $F = 0.10$; $f = 0.25$; $b/R = 0.10$; $\lambda = 0.10$. Curve 4: $F = 0.10$; $f = 0.25$; $b/R = 0.05$; $\lambda = 0.07$. Curve 5: $F = 0.10$; $f = 0.25$; $b/R = 0.02$; $\lambda = 0.04$.

baryons cool, they fall towards the centre to a final mass distribution $M_b(r)$ which, in the case of a spiral galaxy, is constrained by the initial angular momentum. If $F_i \ll 1$, the mass interior to the orbit of a dissipationless particle will not change much during one orbital period even if dissipation occurs rapidly, provided that the particle is far enough from the centre of the galaxy. Thus, the orbits of all but the innermost halo particles change adiabatically under baryonic contraction.

We assume that a fraction $1 - f$ of the initial halo mass decays on a timescale τ_d . The orbit of a halo particle with orbital time $\ll \tau_d$ changes adiabatically. Thus, there is always some inner region of the halo that changes adiabatically under decay and, for $\tau_d \gg t_{dyn}$, the dynamical time of the system, the orbits of all (except, perhaps, the outermost) halo particles obey the adiabatic invariance constraint. After the processes of dissipation and decay are complete, the adiabatic invariant of the dissipationless particle orbits implies⁶

$$r[M_b(r) + M_d(r)] = r_i M_i(r_i) \quad (1)$$

which can be solved for the final DM mass distribution $M_d(r)$ once $M_i(r_i)$ and $M_b(r)$ are given. Here r_i and r are the initial and final orbital radii and $M_d(r) = f(1 - F_i)M_i(r_i)$ (we assume that there is no shell crossing during the contraction of the dissipationless halo⁶). We take the initial mass distribution to be that of an isothermal sphere with core radius $a \equiv 3v^2/(4\pi G\rho_0)$, where v is the one-dimensional velocity dispersion and ρ_0 is the central density. The final radial mass distribution of the baryons is assumed to be $M_b(r) = M_b[1 - (1 + r/b)\exp(-r/b)]$, which describes the mass distribution of a thin disk whose surface density falls off exponentially with scale length b . (For the adiabatic invariance calculation, the baryonic mass is actually treated as if it were distributed spherically rather than in a disk⁶).

After the DM decay is complete, the baryonic mass fraction is $F = M_b/M_i$, where M_i is the final total mass. Figure 1 shows rotation curves resulting from dissipation and decay within an initial isothermal sphere whose core radius is 42% of its outer truncation radius R . We assume that there is no baryonic infall beyond R . The two solid lines are the rotation curves for two values of f and typical values of F and the collapse factor b/R . The ratio b/R is constrained by the initial angular momentum. The mean value of the spin parameter $\lambda \equiv J|E|^{1/2}G^{-1}M_i^{-5/2}$ is estimated by the tidal torque theory¹⁵ to be 0.07 (J , E and M_i are respectively the total angular momentum, energy and mass of a galaxy). Unlike F , which is assumed to be a spatial constant, λ varies among protogalaxies, with a dispersion of ~ 0.03 . This implies that $0.02 \leq b/R \leq 0.1$ (refs 6, 16). The two dotted lines

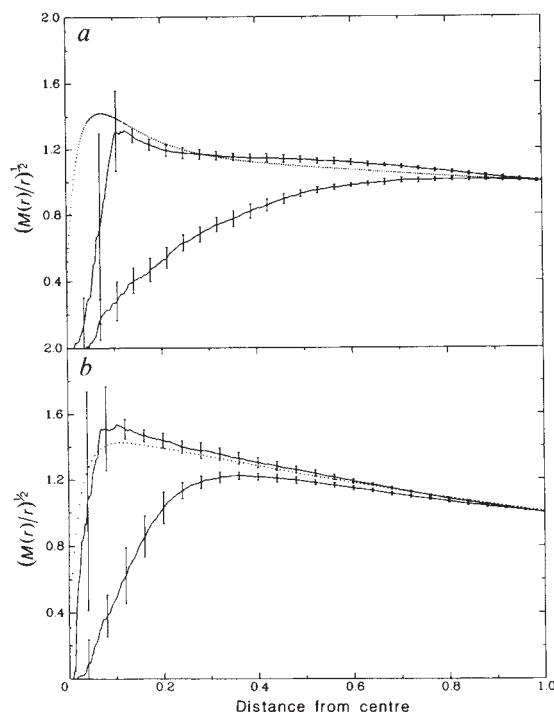


Fig. 2 'Rotation' curves for the numerical models. Ordinate is $[M(r)/r]^{1/2}$, where $M(r)$ is, for every r , an average over several dynamical times after the system reached approximate equilibrium; bars, 1 s.d. fluctuation levels. The abscissa is the distance from the centre in units of the radius that contains 80% of the final total mass (edge effects probably affect the results of the numerical models outside this radius). The dotted lines are the result of the analytical model for $[M(r)/r]^{1/2}$. *a*, Results for a 'hot' start $F = 0.15$; $f = 0.60$; $\lambda = 0.07$. *b*, Results of a Hubble start $F = 0.10$; $f = 0.60$; $\lambda = 0.07$.

in Fig. 1 show the effect of this dispersion on rotation curves: curves 3 and 5 are rotation curves calculated for $f = 0.25$ and $F = 0.1$ with b/R taken at its upper (3) and lower (5) limit. Astrophysical constraints on F place it in the range $0.05 \leq F \leq 0.2$ (ref. 4). The closely dotted line (1) shows the effect on the rotation curve of decreasing F to 0.05 for $f = 0.75$ and $\lambda = 0.07$.

Figure 1 shows that for small f , the velocity decreases at large distances. This can be understood as follows: after the baryons have cooled to an exponential disk (dissipation and decay do not commute because λ varies during the contraction but is invariant under decay in the adiabatic approximation), the DM decay hardly changes $v(r)$ at small radii where the baryons dominate. At large radii, where the DM dominates, $v(r)$ decreases substantially as a result of the system's expansion. Thus, a lower bound on f can arise from the constraint that observed rotation curves do not fall with distance even well outside the optical radius.

The analytical model assumes that the system changes adiabatically, circular orbits, spherical symmetry, and an equilibrium starting configuration. We have relaxed these assumptions and studied more general cases using a dissipative N -body code based on the code developed by Aarseth¹⁷. Particles interact via a newtonian potential softened on small scales to suppress spurious two-body relaxation effects⁶. Integration time steps were chosen so that the fractional change in the total energy is $< 1\%$ over a whole simulation, and the integration continued for several dynamical times, $t_{dyn} \equiv GM_i^{3/2}/|2E|^{3/2}$, after the system reached approximate equilibrium. The system was chosen to have total mass $M_i = 10^{12} M_\odot$, and to consist of $N_f + N_{1-f}$ dissipationless particles and 500 dissipational particles. We chose the number of stable (N_f) and unstable (N_{1-f}) dissipationless particles large enough to have adequate statistics. For

example, for $f=0.4$ we took $N_f=500$ and $N_{1-f}=750$.

The dissipational particles, which were initially mixed uniformly with the DM particles, were assumed to have a collisional cross-section σ , which was kept constant throughout a simulation. A collision occurred if two particles came within a distance $(\sigma/\pi)^{1/2}$ of each other, in which case they were merged to form a single particle, located at their centre of mass and moving with its velocity and mass. Their relative energy was thus dissipated (σ controls the rate of baryonic infall). We chose σ such that the total energy loss rate caused by inelastic collisions equals the physical cooling rate (assuming fully ionized H+He). The decay of the unstable DM was mimicked by randomly selecting N_{1-f} dissipationless particles and assigning them a time $t_{\text{decay}} = -\tau_d \log \theta$, where θ is a random number in the interval (0, 1] chosen for each particle. At time t_{decay} the particle was eliminated from the simulation.

We ran two sets of simulations: (1) 'hot start' models, in which dissipational infall starts from a uniform density sphere in both position and velocity space with the virial condition $Q = KE/|PE| = 0.5$ (kinetic energy/potential energy) satisfied; (2) 'Hubble start' models, which start from a Hubble-expanding homogeneous sphere. In both sets of simulations, angular momentum is imposed by rigid-body rotation. For a discussion of the motivation for these initial conditions as well as the results for the case $f=1$ (no decay) see ref. 6.

Figure 2a shows the results of a hot start with $\lambda=0.07$ and $F=0.15$. The lower solid line is the resulting 'velocity' profile, $[M(r)/r]^{1/2}$ plotted against r , after an unstable fraction $1-f=0.40$ of the initial DM has decayed with a lifetime $\tau_d=3t_{\text{dyn}}$ and no dissipation has taken place. The upper solid line is the resulting profile when the baryons are allowed to dissipate; for the chosen value of σ dissipation is 80% complete by $t=t_{\text{cool}} \sim 3t_{\text{dyn}}$. The dotted line is the result of the analytical model for the same values of F and f , and a collapse factor $b/R=0.05$ corresponding to $\lambda \approx 0.07$; the initial state was taken to be the profile resulting from an N -body simulation without dissipation. The agreement between the two models confirms the validity of the analytical model.

Figure 2b compares the result of a Hubble start with that of the analytical model for $f=0.60$; here $\lambda=0.07$ and $F=0.1$. The DM decays with a lifetime $\tau_d=5t_{\text{dyn}}$, which corresponds to $1+z_d \approx 4$. The initial Hubble constant is chosen so that the protogalaxy expands by a factor of ~ 2.5 before collapse. The meaning of the curves is the same as in Fig. 2a. Again, the agreement between the numerical and analytic profiles is good.

The flat or rising velocity profiles of spiral galaxies¹⁸⁻²¹ are a strong constraint on the models being studied. Figure 1 shows that for typical values of F and λ , the final velocity profile is sensitive to the value of f , and not every value of f yields

acceptable rotation curves. The numerical simulations show that the analytical model works well and therefore can be used to calculate theoretical rotation curves and compare them with observations. The parameters of the analytical model are the final baryon fraction F , the collapse factor b/R , the core radius a/R of the initial isothermal sphere, and the fraction of stable DM f . As Fig. 1 shows, if for a given value of f one obtains a falling velocity profile for F and R/b taken at their lowest values and $a/R \approx 0.4$, then only falling rotation curves can be produced. Figure 3 shows velocity profiles calculated with various core radii for $f=0.5$ and F and R/b taken at their minimum values. Non-decreasing profiles can only arise for $a/R \approx 0.2-0.4$. The range of values of a/R for which non-decreasing rotation curves are obtained narrows as f decreases.

The rotation curves of spiral galaxies are observed to be mostly flat or rising inside the optical radius, with only a small fraction $\sim 20\%$ of the Burstein-Rubin¹⁸ sample observed to have falling velocity profiles. For $f \leq 0.3$, however, we find the theoretical rotation curves to be decreasing inside the optical radius $R_H \equiv 4.5b$ for all values of a/R when F and R/b are at their lowest values. Furthermore, velocity profiles are observed to be flat or rising outside the optical radius as well¹⁹, and this provides further constraints on f . Because we assume f to be a spatial constant, we can use the data of individual galaxies to constrain it. In particular, no value of the initial core radius can yield a rotation curve which is flat out to $r=11b$ (like that of NGC3198 (ref. 20)) unless $f \geq 0.4$, nor can a profile rising out to $r \sim 8b$ (like that of NGC3109 (ref. 21)) be obtained if $f \leq 0.5$. These bounds are, again, for F and R/b at their minimum values.

Thus, rotational profiles of spiral galaxies require that the fraction of stable DM $f \geq 0.5$. The relative contribution by relativistic particles to the energy density is

$$\Omega_r/\Omega_{\text{nr}} \leq f^{-1}(1-f)(1-F)(1+z_d)^{-1} \quad (2)$$

(The equality is satisfied in the instantaneous decay approximation³.) Because $F < 1$, for $f \geq 0.5$ one gets $\Omega_r/\Omega_{\text{nr}} \leq (1+z_d)^{-1}$. Thus, the present Universe could not be dominated by weakly interacting relativistic particles. In the type I models, the relativistic contribution to the energy density required for $\Omega=1$ amounts to $\Omega_r/\Omega_{\text{nr}} \approx 4$. Even for a decay epoch as recent as $1+z_d \approx 5$, equation (2) requires $f \approx 0.05$, a factor of ~ 10 smaller than the bound obtained here. Thus, with the assumptions we have made, the type I model is excluded. One way to relax the assumptions and to decrease the lower limit on f could be to have a substantial portion of the baryonic gas contract dissipatively after the decay, but it is not obvious that such a model can lead to flat rotation curves.

Type II models⁷ do not require that the present Universe be radiation dominated. In these models $\Omega_r/\Omega_{\text{nr}} \approx 0.25$, which is marginally consistent with the upper bound of equation (2) for a decay epoch as recent as $1+z_d \approx 4$. However, an upper bound on f must come from the requirement that on large scales Ω be unity with Ω_{cl} being ~ 0.2 . We are trying to quantify this constraint by studying in detail the formation of clusters and large-scale structure in the decaying DM model.

We thank James Bensinger for the use of the High Energy Physics Vax at Brandeis and R.A.F. thanks Larry Abbott for useful conversations. We also thank the staff of the Aspen Center for Physics for kind hospitality. This work was supported by Department of Energy contract DE-AC02-76ER03230 at Brandeis and NSF grant PHY-8415444 and a grant from the Research Committee at the University of California at Santa Cruz.

Received 1 May; accepted 15 August 1986.

1. Turner, M. S., Steigman, G. & Krauss, L. *Phys. Rev. Lett.* **52**, 2090-2093 (1984).
2. Gelmini, G., Schramm, D. & Valle, J. *Phys. Lett.* **B146**, 311-317 (1984).
3. Turner, M. S. *Phys. Rev. D* **31**, 1212-1224 (1985).
4. Blumenthal, G., Faber, S., Primack, J. & Rees, M. *Nature* **311**, 517-525 (1984).
5. Peebles, P. J. E. *Nature* **321**, 27-32 (1986).
6. Blumenthal, G., Faber, S., Flores, R. A. & Primack, J. *Astrophys. J.* **301**, 27-34 (1986); UCSC Preprint (1986).
7. Olive, K., Seckel, D. & Vishniac, E. *Astrophys. J.* **292**, 1-11 (1985).

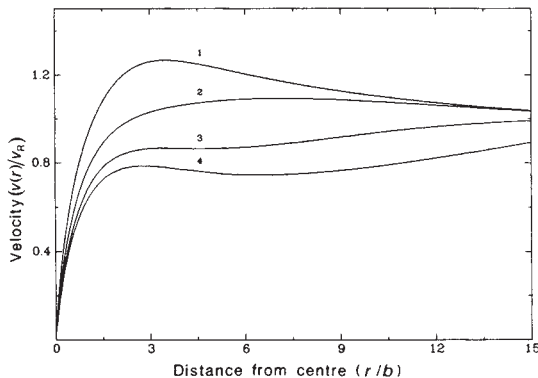


Fig. 3 Rotation curves in the analytical model for various values of the core radius of the initial isothermal sphere. v_R is the velocity at the expanded truncation radius R . $F=0.05$; $f=0.5$; $\lambda=0.1$. Curve 1: $a/R=0.11$. Curve 2: $a/R=0.21$. Curve 3: $a/R=0.42$. Curve 4: $a/R=1.0$.

8. Silk, J. & Vittorio, N. *Phys. Rev. Lett.* **54**, 2269–2272 (1985).
9. Turner, M. S. *Phys. Rev. Lett.* **55**, 549 (1985).
10. Kolb, E. W., Olive, K. & Vittorio, N. Preprint, Fermilab-PUB-86/40-A (1986).
11. Efstathiou, G. *Mon. Not. R. astr. Soc.* **213**, 29P–34P (1985).
12. Dekel, A. & Piran, T. Preprint, Weizmann Institute (1986).
13. Davis, M. & Villumsen, J. V. Preprint, Univ. of California, Berkeley (1985).
14. Hoffman, Y. Preprint UPR-0291-T, Univ. of Pennsylvania (1986).
15. Efstathiou, G. & Jones, B. J. T. *Mon. Not. R. astr. Soc.* **186**, 133–144 (1979).
16. Fall, S. M. & Efstathiou, G. *Mon. Not. R. astr. Soc.* **193**, 189–206 (1980).
17. Aarseth, S. J. in *Multiple Time Scales* (eds Brackbill, J. U. & Cohen, B. I.) 377–418 (Academic, New York, 1985).
18. Burstein, D. & Rubin, V. C. *Astrophys. J.* **297**, 423–435 (1985).
19. Bosma, A. *Astr. J.* **86**, 1791–1824 (1981); **86**, 1825–1846 (1981).
20. van Albada, T. S., Bahcall, J. N., Begman, K. & Sancisi, R. *Astrophys. J.* **295**, 305–313 (1985).
21. Carignan, C. *Astrophys. J.* **299**, 59–73 (1985).

Table 1 Additional objects in the 1146 ± 111 field

α (1950)	δ	Identification
11 h 40 min 58.6 s	+11°07'23"	Galaxy, $z \approx 0.08$
11 h 46 min 00.1 s	+11°07'03"	Galaxy, $z \approx 0.082$
11 h 46 min 02.3 s	+11°08'55"	Galaxy, $z \approx 0.080$
11 h 46 min 05.2 s	+11°05'26"	K star
11 h 46 min 07.7 s	+11°05'15"	Galaxy, $z \approx 0.08$

Table 2 Equivalent widths for C III] and Mg II

C III] 1,909 Å	Mg II 2,798 Å	Date
Quasar B		
—	–17.5	9 April
–20.4	–21.4	5 May
–17.0	–27.0	7 May
–19.6	–16.1	6 June
–19.2	–18.3	Average
Quasar C		
—	–13.6	9 April
–7.9	–12.0	5 May
–6.4	–8.1	7 May
–6.4	–12.3	7 June
–7.0	–12.8	Average

Averages from summed spectra for C III], and from summed spectra + FOGS spectrum weighted by count rate for Mg II. The error in an individual equivalent width measurement is ~15%.

Is 1146+111B, C a lensed quasar or a quasar pair?

J. P. Huchra

Harvard-Smithsonian Center for Astrophysics, 60 Garden Street, Cambridge, Massachusetts 02138, USA

It has been speculated^{1,2} that the quasar pair 1146+B, C are two bright images of a single quasar produced by a gravitational lens. Here I report additional observations of these objects, made with an ultraviolet-sensitive spectrograph on the Multiple Mirror Telescope, which span the wavelength range 3,200–7,000 Å, and include the strong C III] line, as well as several Fe II bands not covered in earlier spectra. The ultraviolet spectra of the two quasars are different. There are also different velocity shifts between the quasars as measured by the C III] and Mg II lines. Although it is impossible to rule out the lensing hypothesis, these observations increase the probability that these objects are just two quasars at nearly the same redshift.

Much interest has been shown in a pair of quasars with nearly identical redshifts ($z \approx 1.01$) discovered by Hazard *et al.*³ in 1979. Although Arp and Hazard⁴ dismissed the possibility that these objects might be the images produced by a gravitational lens because of their very large separation (157 arc s) Paczynski¹ recently suggested that such large splittings could be the results of lensing by cosmic strings. Turner *et al.*² obtained spectra of these objects with a high signal-to-noise ratio using the cryogenic camera at the Kitt Peak National Observatory 4-m telescope. These spectra, which cover the region of the redshifted Mg II line, were quite similar. In addition, the redshift difference between the quasars as measured from the Mg II line was less than the observed error of ~300 km s^{–1}.

Additional spectra of the two objects were obtained by Shaver and Christiani⁵ covering the wavelength region 5,600–9,200 Å. They failed to confirm the similarity of the objects; object B showed much stronger Balmer-line emission than object C.

I have obtained moderate-resolution spectra of 1146+111B and C at several epochs with spectrographs on the Multiple Mirror Telescope (MMT). The first set was obtained on 9 April 1986 (MST) with the MMT faint-object grism spectrograph (FOGS)⁶, and covers essentially the same wavelength range as Turner's² spectra taken one month earlier. These spectra, with a resolution of ~15 Å, confirm the similarity both in shape and redshift of the Mg II line in the two objects. At the same time I obtained spectra of several of the brighter galaxies in the field between or near the objects (Table 1). All appear to be members of a rich group or small cluster of galaxies at a redshift of $z \approx 0.082$.

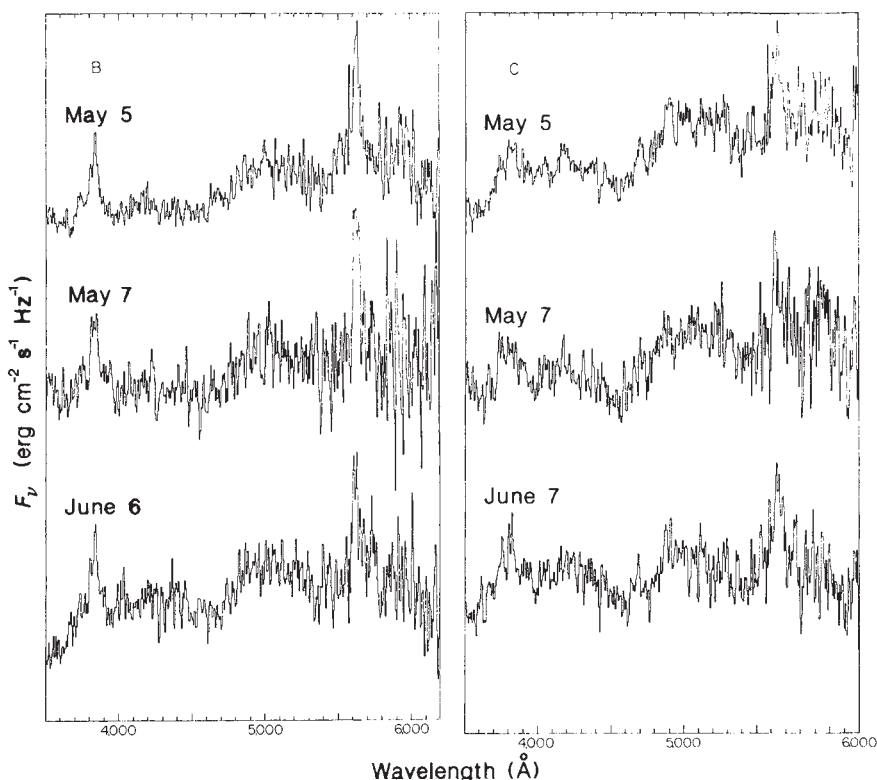
The second, third and fourth sets were obtained with the photon-counting Reticon system on the MMT⁷, on the nights of 5 May (B and C), 7 May (B and C), 6 June (B) and 7 June (C). The intention was to improve the redshift determinations for these objects by observing the C III] 1,909-Å line, which is usually narrower than the Mg II 2,798-Å feature and is less

likely to be variable. Each exposure was ~30 min long and was made through dual 1×3 arc s slits separated by 32 arc s. The astronomical 'seeing' varied from 2.5 arc s on the worst night (7 May) to <1 arc s on the best (7 June). These spectra cover the wavelength range 3,200–7,000 Å, at a resolution of ~9 Å. The spectrograph was carefully focused before these exposures and the focus checked by measurement of the mean comparison and sky line-widths; helium–neon–argon comparison exposures were taken before and after each individual exposure. The [O I] 5,577-Å sky line-width varied by <8% from spectrum to spectrum. The individual exposures are shown in Fig. 1a, b; the summed spectra of the two objects are shown in Fig. 2.

The spectra of the two objects in the vicinity of the Mg II line are similar, in agreement with ref. 2. The spectra of the two objects at <5,000 Å, however, are clearly different. Quasar B has a strong C III] line of equivalent width (EW) –19 Å; quasar C has a much weaker C III] feature of EW –7 Å. Even after correction for continuum contamination by Fe II emission, C's C III] line EW is still only half of B's. In contrast, the Mg II features in the two objects are almost the same: EW ≈ –18 Å in B and –13 Å in C. The equivalent widths of these lines do not vary significantly from these means in the individual spectra (Table 2); the larger scatter in the Mg II widths is due to the difficulty of subtracting the strong [O I] 5,577-Å line and the low efficiency of the MMT spectrograph plus 300 lines per mm grating at 5,600 Å. On the other hand, quasar C shows relatively strong Fe II bands⁷, the 2,100-Å band redshifted to 4,180 Å, the 2,500-Å band at 5,000 Å and the 2,950-Å band at 5,900 Å. These bands are present in quasar B but are much weaker. Quasar C also exhibits the 1,860-Å feature seen just shortward of C III], which is probably due to Al III or Fe emission^{8,9}. This feature is weak in quasar B.

In addition to these results and the spectra in the near-infrared obtained at the European Southern Observatory by Shaver and Christiani⁵, deep charge-coupled-device (CCD) imagery has been obtained at Lowell Observatory by Tyson and Gullixson¹⁰, and at Kitt Peak National Observatory by S. Djorgovski (personal communication). Both the near-infrared spectra of the two objects and their optical broadband colours are different. M. Schmidt (personal communication) has suggested that the

Fig. 1 Individual observations with the MMT spectrograph of objects B (left) and C (right). F_ν is the relative flux. May 7 was the night of worst seeing. These spectra have been Hanning-smoothed twice (the resolution is 3.5 pixels), as have the spectra in Figs 2 and 3.



spectral differences may be due to the presence of extended emission in quasar B. Neither object appears extended in the deep CCD images; the equivalent width differences would require even pure-emission-line objects to be visible at 24 mag. In any case, a visible extent on arc second or larger scales in one object and not in the other is an argument against the lensing hypothesis.

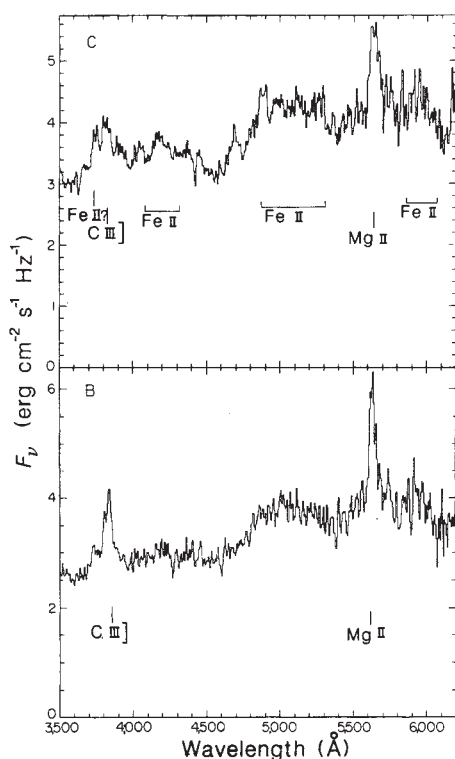


Fig. 2 Summed observations with the MMT spectrograph of objects B and C with features identified.

I have also tried to improve the measurement of the velocity difference between B and C. A simple cross-correlation of the summed MMT-spectrograph spectra of B and C over the entire wavelength range yields a velocity difference ($V_B - V_C$) $\approx +400 \pm 100 \text{ km s}^{-1}$. Cross-correlation of the Mg II line alone yields ($V_B - V_C$) $\approx -100 \pm 60 \text{ km s}^{-1}$. Cross-correlation of the region around C III] alone yields ($V_B - V_C$) $\approx +100 \pm 60 \text{ km s}^{-1}$, although velocities derived in this region can be compromised by variations in the relative strengths of Fe and Al multiplets. Figure 3 shows the regions around the C III] and Mg II lines in both quasars. The different velocity difference between the carbon and magnesium lines is not surprising, especially if B and C are separate quasars. Velocity differences of $1,000 \text{ km s}^{-1}$ are often seen between the lines of different ionization states in the same quasar^{11,12}.

The original arguments for suggesting that 1146+111B, C are images of a lens were based on their nearly identical redshifts and the similarity of their spectra at Mg II. These arguments have weakened as more data have accumulated. Under the double-quasar hypothesis, the similarity of the spectra at Mg II is not that improbable. Quasars of similar luminosity (B and C are $m_B \approx 18.5$ and 18.6 , respectively, at the same redshift) have similar Mg II equivalent widths¹³. It is also well known that quasars are often associated with groups and clusters of galaxies^{14,15}. The velocity difference between the quasars is typical of the velocity dispersions of small groups of galaxies¹⁶. Recent calculations of the probabilities of chance association of quasars, given the total number of quasars that have been observed to date, support the likelihood of finding clustered quasars^{17,18}.

It is unfortunately impossible to rule out the lensing hypothesis with the available data because of the possibly large phase delay between images with such a large separation²—we have no idea what quasars do on thousand-year timescales. Because of the great variability of quasars in brightness, spectral shape and line profiles it is unlikely that any single observation of this pair of objects will give a clear answer to the question of lens or double. However, given the substantial dissimilarity of the spectra of B and C in the ultraviolet and infrared, the

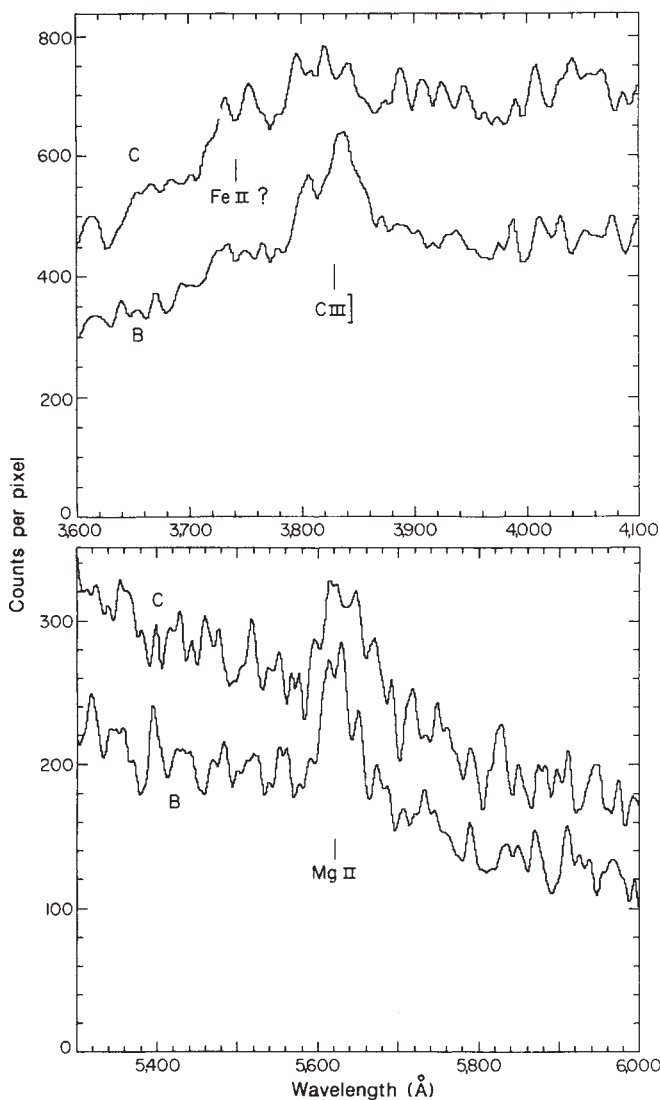


Fig. 3 Enlargements of the raw (counts per pixel) summed spectra of B and C in the regions of C III] (upper) and Mg II. The slight redshift of the Mg II line in B relative to the line in C, and the slight blueshift of the C III] line in B relative to C. The Mg II line in C also appears broader than that in B, but this may be an artefact given the low signal-to-noise ratio of the spectra at 5,600 Å.

different relative velocities of different spectral features and the lack of an identifiable lensing object, this system is more likely to be simply two associated quasars at nearly the same redshift. I thus support the original, simple hypothesis of Arp and Hazard⁴.

I thank Drs F. Chaffee, C. Foltz, E. Turner and B. Wilkes for helpful discussions; John McAfee, Carol Heller and Janet Robertson for efficient handling of the MMT; K. Gilmore for assistance with the FOGS; S. Tokarz and J. Morse for help in data reduction; and Dr Margaret Geller for the use of some of her telescope time. This research has been supported by the Smithsonian Institution. The MMT is a joint facility of the Smithsonian Institution and the University of Arizona.

Received 7 July; accepted 15 September 1986.

1. Paczynski, B. *Nature* **319**, 567–568 (1986).
2. Turner, E. *et al.* *Nature* **321**, 142–144 (1986).
3. Hazard, C., Arp, H. C. & Morton, D. M. *Nature* **282**, 271–272 (1979).
4. Arp, H. C. & Hazard, C. *Astrophys. J.* **240**, 726–736 (1980).
5. Shaver, P. & Christiani, S. *Nature* **321**, 585–586 (1986).
6. Geary, J., Huchra, J. & Latham, D. *Soc. Photo-Optical Instrum. Engr* (in the press).
7. Latham, D. in *Instrumentation for Astronomy with Large Optical Telescopes* (ed. Humphries, C.), 259–270 (Reidel, Dordrecht, 1982).

8. Grandi, S. *Astrophys. J.* **251**, 451–464 (1981).
9. Hartig, G. & Baldwin, J. *Astrophys. J.* **302**, 64–80 (1986).
10. Tyson, T. & Gullixson, C. *Science* (in the press).
11. Gaskell, C. M. *Astrophys. J.* **263**, 79–86 (1982).
12. Wilkes, B. J. *Mon. Not. R. astr. Soc.* **207**, 73–98 (1984).
13. Richstone, D. & Schmidt, M. *Astrophys. J.* **235**, 361–376 (1980).
14. Yee, H. & Green, R. F. *Astrophys. J.* **280**, 79–90 (1984).
15. Stockton, A. *Astrophys. J.* **223**, 747–757 (1978).
16. Huchra, J. & Geller, M. *Astrophys. J.* **257**, 423–437 (1982).
17. Phinney, E. S. & Blandford, R. *Nature* **321**, 569–570 (1986).
18. Bahcall, J., Bahcall, N. & Schneider, D. Preprint.

Is the 1.5-ms pulsar a young neutron star?

John M. Blondin* & Katherine Freese†

* Department of Astronomy and Astrophysics, University of Chicago, Chicago, Illinois 60637, USA

† Institute for Theoretical Physics, University of California, Santa Barbara, California 93106, USA

The 1.5-ms pulsar PSR1937+214 is an unusual object; its extremely short period and slow spin-down rate imply a magnetic field¹ of 4×10^8 G, much lower than that of a canonical pulsar. Contrary to previous models, we propose here, as an explanation for these properties, that PSR1937+214 is a young neutron star (consistent with a kinematic age of $\sim 10^6$ yr) spun up by accretion from a high-mass companion in a close binary system. The supercritical mass transfer rates expected in such a binary system should allow the neutron star to be spun up in the comparatively short time of $\sim 10^4$ yr. The accretion process will also power thermomagnetic effects that could remove the strong magnetic field of a young pulsar from the crust of the star in a similarly short timescale. Such a high-mass binary system is expected to disrupt when the companion explodes in a supernova. Thus a spin-up model in a high-mass system can explain the lack of a companion, low magnetic field, and high spin rate of PSR1937+214.

The discovery of the 1.5-ms pulsar² PSR1937+214 has stimulated much theoretical interest as well as extensive searches for similar objects. Systematic searches over the past few years have led to the discovery of the millisecond pulsars PSR1953+29 (period, $P = 6.1$ ms)³ and PSR1855+09 ($P = 5.4$ ms)⁴ but have not unearthed any additional candidates for isolated millisecond pulsars (no binary companion)^{5,6}. Although the evolution of PSR1937+214 may have been similar to that of the millisecond pulsar binaries, the lack of a present-day companion provides a further constraint on any possible evolutionary scenario.

In the standard model neutron stars are thought to be born spinning rapidly with large dipolar magnetic fields⁷, $B \sim 10^{12}$ G. This field is believed to decay⁸ (possibly through ohmic dissipation) with a lifetime of $\sim 3 \times 10^6$ yr. The pulsar PSR1937+214 is unique in that it has both an extremely short period of 1.56 ms and an exceptionally slow spin-down rate¹, $\dot{P} = 1.2 \times 10^{-19}$ s s⁻¹. A pulsar spinning so rapidly is expected to be a young neutron star, and yet the estimated low magnetic field, $B [\propto (P\dot{P})^{1/2}] \approx 4 \times 10^8$ G, is appropriate to a very old neutron star. Alpar *et al.*⁹ presented a possible resolution of this discrepancy: they suggested that the 1.5-ms pulsar is an old neutron star, originally in a low-mass binary system. In this picture, the pulsar was spun up to its present rapid rotation by accretion from its binary companion, and has since lost this companion.

Because the pulsar is spinning so fast, the magnetic field at the end of this accretion phase must have been very low (as presently observed) to avoid a rapid loss of its rotational energy. If this low magnetic field is explained by the decay of a large initial field, the neutron star must be $\sim 10^8$ yr old. By assuming a typical accretion rate of $\dot{M} \sim 10^{-9} M_{\odot}$ yr⁻¹ as observed in

X-ray binaries, Alpar *et al.*⁹ infer an accretion timescale of $>10^8$ yr to spin the neutron star up to its observed period. This relatively long evolutionary timescale is consistent with a low-mass companion star ($M < 1 M_\odot$), as inferred in the galactic bulge X-ray sources. The problem with this model is that it does not provide an explanation for the apparent lack of a low-mass companion. We see the 1.5-ms pulsar as an isolated object today, and yet a low-mass companion would not have been able to eject enough mass during its final evolution to disrupt the binary system. To resolve this dilemma, Ruderman and Shaham¹⁰ argue that a very light secondary may be tidally disrupted within $\sim 10^{10}$ yr. However, accretion from a very light secondary may not leave a neutron star spinning with a millisecond period¹¹. Alternatively, van den Heuvel and Bonsema¹² postulate the disruption of a massive white dwarf to explain the millisecond pulsar.

Another inconsistency with low-mass models is the low height (z) above the galactic plane of the 1.5-ms pulsar ($z \approx 10$ pc for a heliocentric distance of 2 kpc)¹. This small scale height is typical of (young) high-mass objects, rather than (old) low-mass binary systems. The low z of PSR1937+214 may simply be the result of selection effects in the present pulsar surveys (note that for the two known millisecond binary pulsars, $z < 30$ pc). If PSR1937+214 were born in the galactic plane, this low z suggests a kinematic age of $\sim 10^6$ yr. Although the characteristic age is much longer, $P/2\dot{P} \approx 2 \times 10^8$ yr, this is really only an upper limit and tells us more about the low field of the pulsar than about its historical age.

An alternative explanation for the millisecond pulsar might be that it is a young neutron star ($\tau \approx 10^6$ yr) and was simply born in a supernova with a low original magnetic field^{13,14}. In this case, one is hard pressed to explain why other low-field pulsars have not been found.

We propose an alternative model, wherein the 1.5-ms pulsar is a young object, consistent with the kinematic age of $\tau \approx 10^6$ yr. The pulsar originally had a high-mass binary companion, and either was born with or quickly developed¹⁵ a typical magnetic field of $\sim 10^{12}$ G. A rapid mass transfer phase both spun up the pulsar to the observed rapid rotation and lowered the magnetic field to the inferred value of $\sim 4 \times 10^8$ G. The high-mass companion evolved rapidly and ejected sufficient mass during core collapse to disrupt the binary system, leaving an isolated, low-field pulsar.

In our model, two OB stars evolved in a close binary system. The slightly more massive star went through a supernova phase to become a neutron star orbiting a high-mass, evolved companion. When this companion filled its Roche lobe matter began to fall onto the neutron star. The mass loss from the OB star is expected to be $(10^{-3}-10^{-4}) M_\odot \text{ yr}^{-1}$ for a timespan of $\sim 10^4$ yr (ref. 16). Since this rapid mass transfer phase both spun up the pulsar to the observed rapid rotation and lowered the magnetic field to the inferred value of $\sim 4 \times 10^8$ G. The high-mass companion evolved rapidly and ejected sufficient mass during core collapse to disrupt the binary system, leaving an isolated, low-field pulsar.

Such high accretion rates are supercritical; they surpass the accretion rate associated with the Eddington luminosity of a neutron star, $\dot{M}_E = L_E R_{ns} / (GM_{ns}) = 4\pi R_{ns} c / \kappa \approx 1.5 \times 10^{-8} M_\odot \text{ yr}^{-1}$, where R_{ns} is the neutron star radius and κ is the radiative opacity (assumed to be due to electron scattering). In the case of spherically symmetric accretion, one might therefore worry that the radiation pressure of the accretion-driven luminosity would be high enough to blow most of the infalling material away from the star. However, as in the cauldron model of Begelman and Rees¹⁷, the infalling matter may deviate from spherical symmetry and fall to the surface in clumps, with the excess super-Eddington luminosity being used

to expel some fraction of the infalling matter in the form of bipolar jets. In any case, although a complete theory of supercritical accretion does not yet exist, there is observational evidence for this phenomenon in the relativistic-jet source SS433¹⁸. Van den Heuvel¹⁹ argues that SS433 is a binary system with a massive companion in a phase of rapid mass transfer with $\dot{M} \approx 10^{-4} M_\odot \text{ yr}^{-1}$. If the compact object is a young neutron star, this system would be similar to the progenitor system of PSR1937+214 suggested by our evolutionary model.

In addition to spinning up the star, supercritical accretion will have strong effects on the thermal structure of the neutron star crust. As the accreting material is compressed in the envelope of the star, it approaches sufficiently high densities that nuclear burning will occur. This energy release will heat the base of the envelope to temperatures of $\sim 10^8-10^9$ K. Because the core of the neutron star is held to temperatures of $< 10^8$ K by neutrino cooling, a temperature gradient develops between the surface and the core of the star. The heat flux into the star, F_{in} , driven by this temperature gradient is a monotonically increasing function of the accretion rate^{20,21}. The neutrino cooling can effectively maintain a net inward heat flux only for the supercritical accretion rates independently required in our model. As long as $\dot{M} < GM_{ns} \dot{M}_E / (R_{ns} c^2 q) \approx 3 \times 10^{-7} M_\odot \text{ yr}^{-1}$ (where $q \approx 10^{-2}$ is the fraction of the rest mass energy released in nuclear burning), a static envelope exists, and one can calculate²¹ $F_{in}(\dot{M})$. An accretion rate of $\dot{M} = 2.1 \times 10^{-7} M_\odot \text{ yr}^{-1}$ will heat the base of the envelope to $T = 1.4 \times 10^9$ K (I. Fushiki, personal communication). Using the analytic expressions of Hernquist and Applegate²², we find that this $\dot{M}$ corresponds to an accretion driven heat flux of $F_{in} \approx 5 \times 10^{22} \text{ erg cm}^{-2} \text{ s}^{-1}$. For larger values of $\dot{M}$ the nuclear energy released exceeds the Eddington luminosity and a static envelope solution does not exist. In this case, we assume that larger values of $\dot{M}$ will continue to drive a heat flux which is at least as large as $5 \times 10^{22} \text{ erg cm}^{-2} \text{ s}^{-1}$.

If the magnetic field, B , is anchored in the crust of the neutron star (see ref. 15), such a heat flux will power thermomagnetic effects that can reduce the magnitude of the field. The evolution of magnetic fields in the crust of a neutron star is given by the induction equation,

$$\frac{\partial \mathbf{B}}{\partial t} = -\nabla \times \mathbf{E} \\ = \nabla \times (\mathbf{V} \times \mathbf{B}) - \nabla Q_0 \times \nabla T - \nabla \times (\nabla \times \eta \mathbf{B}) \quad (1)$$

where Q_0 is the thermopower, $\eta \equiv c^2 / (4\pi\sigma_0)$, and σ_0 is the electrical conductivity. In the limit that $\mu_e^2 \gg m_e^2 c^4$, where μ_e is the electron chemical potential, Q_0 is given by $Q_0 \approx -\pi^2 k_B^2 T c / 3\mu_e e$. The first term on the right-hand side represents a convection of the field at a velocity given by the sum of the hydrodynamic velocity, the Hall drift velocity, and the effective thermal drift velocity²³. The second term is the battery term, proportional to $\nabla n_e \times \nabla T$, and the last term represents ohmic diffusion. Other terms resulting from the rotation of the star could, in principle, contribute to equation (1). The Biermann battery term describing the distortion of the star caused by rotation (so that $\nabla n_e \times \nabla T \neq 0$) and the Coriolis force exerted on the conduction electrons moving with the thermal drift velocity are both negligible for fields $B > 10^4$ G.

The inward heat flux resulting from accretion may power the first two terms in equation (1). The Lorentz force acting on thermal electrons moving into the star in the presence of a horizontal magnetic field gives rise to a horizontal heat flux. If the horizontal field is not uniform in the direction of the perturbed heat flux, horizontal temperature gradients will form perpendicular to the vertical density gradients, thereby powering a non-zero battery (second) term¹⁵ in equation (1). Even if there are no horizontal gradients to produce a battery term, the deflection of the conduction electrons will still induce a horizontal thermoelectric field with a nonvanishing curl, thereby driving

the convective (first) term in equation (1). As these two terms are of comparable strength, we assume no horizontal field gradients and simply calculate the effects of the convective term.

In the limit $B \ll B_1 \approx 10^{12} z_4 T_8 g_{14} \text{ G}$, at a depth of $10^4 z_4 \text{ cm}$, a temperature of $10^8 T_8 \text{ K}$, and a surface gravity of $10^{14} g_{14} \text{ cm s}^{-2}$, the motions of the electrons are dominated by scattering processes rather than by the magnetic field^{15,23} and equation (1) can be solved in an approximation linear in B . Our calculations of the field strength indicate that most of the evolution in B will take place in this low-field limit. In this limit, for an inward heat flux, the effective thermal drift convects the magnetic field up to the surface of the star with a velocity²³ of $v_{td} \approx -F_{in}/(n_e \mu_e)$, where n_e is the electron number density. Once the magnetic field reaches the liquid metal ocean above the solid crust, ohmic dissipation and magnetic buoyancy will remove the field on a short timescale of the order of 10 yr.

We can estimate the timescale for the removal of B from the crust to the ocean by thermomagnetic field convection by assuming a smooth, horizontal magnetic field varying only with depth in the crust of the neutron star and a purely vertical temperature gradient. In the low-field limit, the induction equation then reduces to

$$\frac{\partial B}{\partial t} = \frac{\partial}{\partial z} \left(\frac{F_{in} B}{m_e \mu_e} \right) + \frac{\partial}{\partial z} \left(\eta \frac{\partial B}{\partial z} \right) \quad (2)$$

where we have assumed a plane-parallel geometry. The first term on the right-hand side represents the effective thermal drift and the second term represents the ohmic diffusion. The ratio of these two terms is given by the effective thermomagnetic Reynolds number, $R_{TM} = v_{td} l / \eta$, where l is the characteristic length scale. In this case $R_{TM} \propto F_{in}$.

When $R_{TM} \ll 1$, the magnetic field decays on the ohmic timescale given by $\tau_{ohm} = 4\pi\sigma_0 l^2 / c^2$. In our model, we expect $F_{in} \gg 5 \times 10^{20} T_8^2 g_{14} \text{ erg cm}^{-2} \text{ s}^{-1}$, corresponding to $R_{TM} \gg 1$. In this case, we can neglect the ohmic decay term to find the solution,

$$B(z, t) = B_0 z^4 \exp \left[-\frac{4}{5} \left(\frac{z}{z_B} \right)^5 \right] \exp \left(-\frac{t}{\tau_{TM}} \right) \quad (3)$$

where z_B is the depth corresponding to the highest amplitude of magnetic field, and

$$\tau_{TM} = \left(\frac{n_e \mu_e z}{F_{in}} \right)_{z=z_B} = 6 \times 10^3 \left(\frac{z_B}{200 \text{ m}} \right)^5 \left(\frac{F_{in}}{10^{22} \text{ erg cm}^{-2} \text{ s}^{-1}} \right)^{-1} \text{ yr} \quad (4)$$

is the lifetime of magnetic field in the neutron star crust. If we assume $z_B \sim 200 \text{ m}$ (ref. 15) and a maximum magnetic dipole strength of the star of $\sim 10^{30} \text{ G cm}^3$, equation (3) gives a maximum field of $\sim 5 \times 10^{13} \text{ G}$ in the crust. As this is $\sim B_1$, the low-field limit is a good approximation. For this simple geometry the results do not change significantly in the high-field regime. We therefore expect any magnetic field anchored in the crust of a supercritically accreting neutron star to decrease with an exponential timescale of $\sim 10^3$ – 10^4 yr. In the case of PSR1937+214, this process would have ended with a neutron star spinning with a period of 1.56 ms and a surface magnetic field of $\sim 4 \times 10^8 \text{ G}$.

This supercritical accretion phase will terminate when the companion loses all its hydrogen envelope or when the system enters a common-envelope phase²⁴. In either case, the end state will be a neutron star in a tight orbit around the helium core of the companion star. If the evolved core is $> 4.2 M_\odot$, the companion may eject sufficient mass during the core collapse supernova to disrupt the binary system. In this case, the newborn neutron star and the spun-up pulsar will both have runaway

velocities $> 100 \text{ km s}^{-1}$. Thus, we expect the 1.5-ms pulsar to be moving with a high space velocity. The absence of an observable supernova remnant is consistent with this model if the supernova explosion occurred $\geq 10^5$ yr ago.

The phase of rapid mass transfer (RMT) that drives the evolutionary process described here may apply to most massive X-ray binaries. The final state of these systems will depend sensitively on the initial period and mass of the binary system and may only produce millisecond pulsars on rare occasions. For orbital periods larger than a few days the RMT phase may end quickly as the neutron star will soon be engulfed in the expanding envelope of the evolving companion. For very short periods, the orbit decay caused by RMT may also result in the neutron star quickly entering the envelope of its main sequence companion. During this common envelope phase the neutron star will spiral in on a timescale of $\sim 10^3$ yr (ref. 25), possibly too short for any significant reduction of the magnetic field. Alternatively, the RMT phase may annihilate B completely, or reduce it to the constant residual field^{26,27} of $\sim 10^8 \text{ G}$, but not spin it up sufficiently to be observable as a pulsar. There are many such possible final scenarios, but without more detailed knowledge of high-mass binary evolution and of the effects of supercritical accretion on neutron stars, one cannot accurately predict the number of millisecond pulsars expected in this model.

This phase of supercritical accretion spin-up accompanied by thermomagnetic destruction of the field may also have application to the evolution of binary pulsars. The dipole fields of these objects are distinctly lower than that of a canonical pulsar. These systems are explained²⁸ as the end state of a binary system containing a neutron star and a normal companion of mass $M \approx 1$ – $8 M_\odot$. The mass transfer rates in such systems may reach^{29,30} $\dot{M} \approx 10^{-7}$ – $10^{-5} M_\odot \text{ yr}^{-1}$ for 10^5 – 10^6 yr, sufficient to reduce the dipole field by a few orders of magnitude.

We thank J. Arons, R. Blandford, G. Field, I. Fushiki, D. Lamb, S. Rappaport, R. Taam, N. Wingreen and especially A. Königl for useful discussions. J.B. gratefully acknowledges the hospitality of ITP at Santa Barbara. Supported by NSF grants AST84-51727 and AST85-03093 to J.B. and PHY82-17853 with supplemental support from NASA to K.F.

Received 2 May; accepted 13 August 1986.

1. Backer, D. C., Kulkarni, S. R. & Taylor, J. H. *Nature* **301**, 314–315 (1983).
2. Backer, D. C., Kulkarni, S. R., Heiles, C., Davis, M. M. & Gross, W. M. *Nature* **300**, 615–618 (1982).
3. Boriakoff, V., Bucccheri, R. & Fauci, F. *Nature* **304**, 417–419 (1983).
4. Segelstein, D. J., Taylor, J. H., Rawley, I. A. & Stinebrin, D. R. *IAU Circ. No.* 4162 (1986).
5. Stokes, G. H., Taylor, J. H., Weisberg, J. M. & Dewey, R. J. *Nature* **317**, 787–788 (1985).
6. Clifton, T. R. & Lyne, A. G. *Nature* **320**, 43–45 (1986).
7. Manchester, R. N. & Taylor, J. H. in *Pulsars* (Freeman, San Francisco, 1977).
8. Ostriker, J. P. & Gunn, J. E. *Astrophys. J.* **157**, 1395–1417 (1969).
9. Alpar, M. A., Cheng, A. F., Ruderman, M. A. & Shaham, J. *Nature* **300**, 728–730 (1982).
10. Ruderman, M. A. & Shaham, J. *Astrophys. J.* **289**, 244–246 (1985).
11. Jeffrey, L. C. *Nature* **319**, 384–386 (1986).
12. van den Heuvel, E. P. J. & Bonsema, P. F. J. *Astr. Astrophys.* **139**, L16–L18 (1984).
13. Brecher, K. & Channugan, G. *Nature* **302**, 124–125 (1983).
14. Arons, J. *Nature* **302**, 301–305 (1983).
15. Blandford, R. D., Applegate, J. H. & Hernquist, L. *Mon. Not. R. astr. Soc.* **204**, 1025 (1983).
16. van den Heuvel, E. P. J. & DeLoore, C. *Astr. Astrophys.* **25**, 387–395 (1973).
17. Begelman, M. C. & Rees, M. J. *Mon. Not. R. astr. Soc.* **206**, 209 (1984).
18. Königl, A. *Mon. Not. R. astr. Soc.* **205**, 471–485 (1983).
19. van den Heuvel, E. P. J. *Vistas Astr.* **25**, 95–108 (1981).
20. Ayasli, S. & Joss, P. C. *Astrophys. J.* **256**, 637–665 (1982).
21. Fujimoto, M. Y., Hanawa, T., Iben, I. & Richardson, M. B. *Astrophys. J.* **278**, 813–824 (1984).
22. Hernquist, L. & Applegate, J. H. *Astrophys. J.* **287**, 244–254 (1984).
23. Urpin, V. A. & Yakovlev, D. G. *Soviet Astr.* **24**, 425–431 (1980).
24. van den Heuvel, E. P. J. in *Accretion-Driven Stellar X-ray Sources* (eds Lewin, W. H. G. & van den Heuvel, E. P. J.) 303–341 (Cambridge University Press, 1984).
25. Taam, R. E., Bodenheimer, P. & Ostriker, J. P. *Astrophys. J.* **222**, 269–280 (1978).
26. van den Heuvel, E. P. J., van Paradijs, J. A. & Taam, R. E. *Nature* **322**, 153–155 (1986).
27. Kulkarni, S. R. *Astrophys. J.* **306**, L85–L89 (1986).
28. van den Heuvel, E. P. J. & Taam, R. E. *Nature* **309**, 235–237 (1984).
29. van der Linden, T. J. *IAU Symp. No.* 88, 109–120 (1980).
30. deKool, M., van den Heuvel, E. P. J. & Rappaport, S. A. *Astr. Astrophys.* (in the press).

Orbital acceleration and the energy budget in the galilean satellite system

Richard Greenberg*, S. J. Goldstein Jr†
& K. C. Jacobs‡

* Lunar and Planetary Laboratory, University of Arizona, Tucson, Arizona 85721, USA

† Department of Astronomy, University of Virginia, Charlottesville, Virginia 22903, USA

‡ Department of Physics, Hollins College, Roanoke, Virginia 24020, USA

Prodigious amounts of heat are generated in Jupiter's satellite Io, as evidenced by its spectacular volcanism¹ and infrared heat flux²⁻⁶ ($\sim 7.6 \times 10^{13}$ W (ref. 7)). Such heating had been predicted from tidal flexing of Io due to the eccentricity of its orbit⁸, which is maintained by its resonant gravitational interaction with the satellites Europa and Ganymede. The energy for tidal heating must come from Io's orbit, but due to the resonance the energy loss is shared among the three satellites' orbits^{9,10}. This effect modifies the rate at which Io's semi-major axis would be expected to decrease, and also tends to drive the system out of resonance. Yoder⁹ suggested that the resonance is maintained by tides raised on Jupiter by Io, which act to transfer planetary rotational energy and angular momentum into Io's orbit, but the jovian torque may be insufficient for this purpose. Greenberg¹⁰ proposed that with smaller jovian torque the system may be evolving away from resonance, and that measurement of Io's orbital acceleration could show whether the evolution is in fact dominated by heating in Io. An acceleration value $\dot{n}_1/n_1 = 4.6 \times 10^{-10} \text{ yr}^{-1} \pm 20\%$, where n_1 is Io's mean motion, derived¹¹ from historical records of Io's motion, is in accord with the expected value $\dot{n}_1/n_1 = 3.2 \times 10^{-10} \pm 28\%$ based on the measured thermal radiation and the assumption of negligible jovian torque. A much smaller negative acceleration has been determined by Lieske^{12,13}, which would imply significant jovian torque, but not enough to maintain the equilibrium proposed by Yoder, unless the average tidal disruption in Io is much less than is indicated by the thermal flux.

The Laplace resonance among the three galilean satellites Io (1), Europa (2) and Ganymede (3) is due to the near-half-commensurability of the orbital periods of the pairs Io-Europa and Europa-Ganymede. In terms of orbital mean motions n (angular velocity), the combinations $n_1 - 2n_2$ and $n_2 - 2n_3$ are both equal to the same small quantity ν . For comparison, n_1 is 203.5° per day and ν is only $\sim 0.8^\circ$ per day. Thus,

$$n_1 - 3n_2 + 2n_3 = 0 \quad (1)$$

or in terms of the integral of equation (1), the mean longitudes obey the relation

$$\lambda_1 - 3\lambda_2 + 2\lambda_3 = 180^\circ \quad (2)$$

The effect of the resonance is to maintain the left side of equation (2) at the stable constant value of 180° , to maintain equation (1) and to force eccentricities in the motion of each satellite that are inversely proportional to ν . This forced eccentricity of Io maintains the tidal heating; otherwise the tidal dissipation would damp the eccentricity¹⁴. The value of ν is a measure of closeness to exact resonance: for small ν , the system is deep in resonance, with correspondingly large orbital eccentricities.

To relate energy loss to shrinkage of the orbit or acceleration of mean motion we use the expression for orbital energy:

$$E = -\frac{1}{2}GMm/a = -\frac{1}{2}m(GMn)^{2/3} \quad (3)$$

where G is the gravitational constant, M is Jupiter's mass, m is the satellite's mass and a is the orbital semi-major axis, related to n by Kepler's third law. The rate of orbital energy

Table 1 Determinations of orbital acceleration (in units of 10^{-10} yr^{-1})

	Io ($\dot{n}_1/n_1$)	Europa ($\dot{n}_2/n_2$)	Ganymede ($\dot{n}_3/n_3$)
From historical observations of motion			
DeSitter ¹⁶	3.3 ± 0.5	2.7 ± 0.7	1.5 ± 0.6
Goldstein & Jacobs ¹¹	4.6 ± 0.9	—	—
Goldstein & Jacobs ¹¹ , based on ref. 18	5.1 ± 1.5	4.2 ± 1.3	-7.6 ± 5.7
Lieske ¹²	-0.074 ± 0.087	—	—
Computed here from Io heat loss			
Negligible jovian torque model	$3.20 \pm 0.89^*$	$1.60 \pm 0.45^\dagger$	$-1.60 \pm 0.45^\ddagger$
Resonance equilibrium	$-1.16 \pm 0.31^\S$	$-1.16 \pm 0.31^\parallel$	$-1.16 \pm 0.31^\parallel$

* From equation (10).

† From equation (12).

‡ From equation (13).

§ From equation (11).

|| From equations (8) and (9): $\dot{n}_1/n_1 = \dot{n}_2/n_2 = \dot{n}_3/n_3$.

change is thus

$$\dot{E} = -\frac{1}{3}m(GM)^{2/3}n^{-1/3}\dot{n} \quad (4)$$

or $\dot{E}/E = \frac{2}{3}\dot{n}/n$. For Io, the tidal heat loss cannot be equated to expression (4) because the energy loss is shared among the orbits of the three resonant satellites:

$$\dot{E}_{\text{tidal}} = -\frac{1}{3}(GM)^{2/3} \sum_{i=1}^3 m_i n_i^{-1/3} \dot{n}_i \quad (5)$$

For the moment we assume that Jupiter exerts no torque on the satellites' orbits.

We wish to express $\dot{E}_{\text{tidal}}$ in terms of the observable $\dot{n}_1$ only. The dependence on $\dot{n}_2$ and $\dot{n}_3$ on the right side of equation (5) can be eliminated by invoking the properties of the resonance and conservation of angular momentum, as follows (see also ref. 15): From the expression for orbital angular momentum L , we have, for the system,

$$L = \sum_{i=1}^3 m_i \sqrt{GMa_i(1-e_i^2)} = \text{constant} \quad (6)$$

where e is the eccentricity. By Kepler's third law, a_i can be expressed in terms of n_i , and because of the resonance, e_i is a function of ν . Thus, differentiation of equation (6) gives

$$f(\dot{n}_1, \dot{n}_2, \dot{n}_3, \dot{\nu}) = 0 \quad (7)$$

where f is a linear function of the four rates. We also have from the resonance relation and the definition of ν :

$$\dot{n}_1 - 2\dot{n}_2 = \dot{\nu} \quad (8)$$

$$\dot{n}_2 - 2\dot{n}_3 = \dot{\nu} \quad (9)$$

Solution of equations (7), (8) and (9) gives the three quantities $\dot{n}_2$, $\dot{n}_3$ and $\dot{\nu}$ in terms of $\dot{n}_1$. With these solutions for $\dot{n}_2$ and $\dot{n}_3$ substituted into equation (5), we have the required relation between $\dot{E}_{\text{tidal}}$ and $\dot{n}_1$, which with appropriate numerical values takes the simple form

$$\dot{E}_{\text{tidal}} = 0.56E_1 \dot{n}_1/n_1 \quad (10)$$

not very different from equation (4). With substitution of the value $\dot{n}_1/n_1 = 4.6 \times 10^{-10} \text{ yr}^{-1} \pm 20\%$ reported in ref. 11, the right side of equation (10) becomes $(1.09 \pm 0.22) \times 10^{14}$ W. Given the range of uncertainty, this value is consistent with the infrared heat flux from Io, $0.76 \times 10^{14} \text{ W} \pm 28\%$ (ref. 7).

Yoder⁹ has suggested that tides raised on Jupiter by Io induce sufficient torque that $\dot{\nu}$ is zero; that is, the system is in stable equilibrium, neither evolving away from exact resonance ($\dot{\nu} > 0$) nor toward it ($\dot{\nu} < 0$). In that case, the above analysis must be modified by setting $\dot{\nu} = 0$, by introducing a rate of change of L

due to the jovian torque, and by including in $\dot{E}_{\text{tidal}}$ (equation (5)) terms for tides in both Io and Jupiter. With these changes we find

$$\dot{E}_{\text{tides in Io}} = -1.54E_1\dot{n}_1/n_1 \quad (11)$$

(compare equations (4) and (10)). By its negative sign, this expression shows that this equilibrium model is discordant with the recent evaluation¹¹ of $\dot{n}_1/n_1$. Given the observed heat flux, this equilibrium model would require a value of $\dot{n}_1/n_1$ comparable in magnitude to that of ref. 11, but of opposite sign (see Table 1). As shown above, the recently determined value¹¹ of $\dot{n}_1/n_1$ is quantitatively consistent with the measured heat flux from Io if we assume that the jovian torque is negligible.

The recent determination¹¹ of $\dot{n}_1/n_1$ also agrees reasonably well with the value obtained much earlier by DeSitter¹⁶, $\dot{n}_1/n_1 = 3.3 \times 10^{-10} \text{ yr}^{-1} \pm 15\%$. DeSitter was sceptical of his own results because, by analogy with the Earth-Moon system, he expected tides raised on Jupiter to decrease n_1 (that is, to drive Io outward, away from the planet) rather than increase it¹⁷. Now, with tidal heating in Io so graphically apparent, the positive $\dot{n}_1$ seems not only reasonable but of just the right magnitude, assuming negligible jovian torque.

DeSitter's values for the acceleration of Europa and Ganymede do not fit this tidal model so well. From equations (7), (8) and (9) we can express $\dot{n}_2$ and $\dot{n}_3$ in terms of $\dot{n}_1$. Thus, we have

$$\dot{n}_2/n_2 = \frac{1}{2}\dot{n}_1/n_1 \quad (12)$$

and

$$\dot{n}_3/n_3 = -\frac{1}{2}\dot{n}_1/n_1 \quad (13)$$

DeSitter's value of $\dot{n}_2/n_2 = 2.7 \times 10^{-10} \text{ yr}^{-1} \pm 25\%$ only marginally satisfies this constraint, and his value $\dot{n}_3/n_3 = 1.5 \times 10^{-10} \text{ yr}^{-1} \pm 40\%$ fails completely. Note, however, the large error bracket associated with the last value; if the actual error is a few times DeSitter's estimate, the determination of $\dot{n}_3$ would not be significant. Thus DeSitter's values for $\dot{n}_2$ and $\dot{n}_3$ are inconclusive as a test of agreement with this tidal theory.

Similarly, if the more recent analysis¹¹ of the 300-yr data set for Io's acceleration could be extended to Europa and Ganymede, it would provide a critical test. Unfortunately, a statistical estimate of the potential accuracy of such results is not encouraging, because of the lower frequency and poorer quality of measurements compared with those for Io, especially in the early (17th century) data.

Apparent support for the recently determined value¹¹ of the acceleration of Io, which was based on 17th century and early 20th century data, comes from the fact that late 19th century observations and theory of the satellites' motion can be reconciled with eclipse observations in the mid-20th century only by applying a time correction for each satellite^{18,19}. In ref. 11 this is interpreted as being due to orbital acceleration, yielding $\dot{n}_1/n_1 = 5.1 \times 10^{-10} \text{ yr}^{-1} \pm 30\%$, $\dot{n}_2/n_2 = 4.2 \times 10^{-10} \text{ yr}^{-1} \pm 30\%$ and $\dot{n}_3/n_3 = -7.6 \times 10^{-10} \text{ yr}^{-1} \pm 75\%$. The first value agrees with the other determinations in ref. 11, and all three are consistent with the heat loss from Io under the assumption of negligible jovian torque (see Table 1). However, the timing corrections might be due to inadequacies in the orbital theory, rather than to orbital acceleration¹².

The several independent methods of determining $\dot{n}_1$ described in ref. 11 give mutually consistent results; however, each method involves controversial elements. For example, the 17th century eclipse timings were measured in Universal Time (UT), essentially using the Earth's rotation as a clock. However, the Earth's rotation rate is variable, so that an evaluation is required for the correction ΔT of UT with respect to the more uniform timescale Ephemeris Time (ET); ET is based on planetary orbital motion. In ref. 11 a formula for ΔT was adopted, based on the assumption of uniform deceleration of the Earth's rotation²⁰. The magnitude of that deceleration was quantified by comparing 19th century theories of the Earth's orbital motion, which were based on UT observations then, with present positions. The value is also reasonably consistent with values of ΔT measured during

the past thirty years by atomic clocks.

On the other hand, Lieske¹² believes that the Earth's rotation is too irregular for the above method to be valid. He prefers to use the lunar orbit as a clock to relate UT to ET, a procedure which involves the assumption that lunar orbital theory is sufficiently accurate and that lunar tidal deceleration is uniform. (The result in ref. 11 is not dependent on lunar theory.) The lunar deceleration rate is obtained from a comparison of lunar motion in the past century with the motion of other planets (the ET clock), and more recently by lunar laser ranging measurements. With this method Lieske¹² finds that $\dot{n}_1$ is negative and much smaller than the values discussed above (see Table 1).

As seen in Table 1, Lieske's value for $\dot{n}_1$ is much too small to agree with the infrared flux, assuming either negligible jovian torque (equation (10)) or resonance equilibrium (equation (11)). In order to reconcile his result with the equilibrium model, he suggests¹³ that the heat flux from Io measured over the past decade is anomalously high compared with the previous 300 yr. The long-term average tidal dissipation rate would need to be an order of magnitude slower than indicated by the present thermal flux, which would require great geophysical changes in a remarkably short time. Alternatively, one could reconcile Lieske's value of $\dot{n}_1$ with the high heat flux if dissipation in Jupiter were $\sim 2/3$ the amount needed for the equilibrium state suggested by Yoder⁹, in which case the system would be evolving outward from exact commensurability (toward larger values of ν)¹⁰.

Goldstein and Jacobs¹¹ value for $\dot{n}_1$ suggests that tidal heating on Io dominates the evolution, with the implication that ν is increasing even faster (by a factor of 2-3) than implied by Lieske's value of $\dot{n}_1$. An objection to that idea^{15,21} is that the system would have been very deep in resonance (small ν) quite recently compared with the age of the Solar System ($<10^8$ yr ago); and relation (2) is unstable there¹⁵. However, there are stable paths for evolution in deep resonance; they involve values of the right side of equation (2) other than 180° (ref. 22). Perhaps the system has recently passed through deep resonance, where Io's eccentricity and tidal heating became large, and is moving out of resonance now, but such a model, which requires a recent special event, is not very satisfying. Perhaps the most plausible model for the long-term history of the system involves an episodic heating-cooling cycle for Io corresponding to periodic variation in ν on a timescale of $\sim 10^8$ yr^{10,23}. This non-equilibrium model can accommodate either determination of $\dot{n}_1$, as well as the present high heat flux from Io. According to this theory, although the system is now rapidly evolving out of resonance, eventually Io's orbital eccentricity and tidal heating will decrease. Then the system will return to deep resonance, where heating will begin again. This cycle may have occurred repeatedly throughout the lifetimes of the galilean satellites. We thank Dr Jay Lieske and Professor Stanton Peale for helpful discussions. K.C.J. acknowledges the support of research and travel grants from Hollins College. R.G. is supported by grant NAGW-944 from the NASA Planetary Geology and Geophysics Program.

Received 15 May; accepted 9 September 1986.

- Morabito, L. A., Synnott, S. P., Kupferman, P. N. & Collins, S. A. *Science* **204**, 972 (1979).
- Hanel, R. *et al. Science* **204**, 972-976 (1979).
- Sinton, W. M. *et al. Science* **210**, 1015-1017 (1980).
- Morrison, D. & Telesco, C. M. *Icarus* **44**, 226-233 (1980).
- Matson, D. L. *et al. J. geophys. Res.* **86**, 1664-1672 (1981).
- Johnson, T. V. *et al. Science* **226**, 134 (1984).
- McEwen, A. S., Matson, D. L., Johnson, T. V. & Soderblom, L. A. *J. geophys. Res.* **90**, 12345-12379 (1985).
- Peale, S. J. *et al. Science* **203**, 892-894 (1979).
- Yoder, C. F. *Nature* **279**, 767-770 (1979).
- Greenberg, R. in *Satellites of Jupiter* (ed. Morrison, D.) 65-92 (University of Arizona Press, Tucson, 1982).
- Goldstein, S. J. & Jacobs, K. C. *Astr. J.* **92**, 199-202 (1986).
- Lieske, J. H. Preprint. *Jet. Prop. Lab.* (1986).
- Lieske, J. H. in *Relativity in Celestial Mechanics and Astronomy* (eds Kovalevsky, J. & Blumberg, V. A.) 117-125 (Reidel, Dordrecht, 1986).
- Goldreich, P. *Mon. Not. R. astr. Soc.* **126**, 257-268 (1963).
- Yoder, C. F. & Peale, S. J. *Icarus* **47**, 1-35 (1981).

16. DeSitter, W. *Leiden Ann.* **16**, Pt 2 (1928).
17. DeSitter, W. *Mon. Not. R. astr. Soc.* **91**, 706-738 (1931).
18. Peters, C. F. *Astr. J.* **78**, 951-956 (1973).
19. Ferraz-Mello, S. *Cel. Mech.* **12**, 27-37 (1975).
20. Goldstein, S. J. *Astr. J.* **90**, 1900-1905 (1985).
21. Peale, S. J. & Greenberg, R. *Proc. lunar planet Sci. Conf.* **11**, 871-873 (1980).
22. Greenberg, R. *Bull. Am. astr. Soc.* **17**, 921-922 (1985).
23. Ojakangas, G. W. & Stevenson, D. J. *Icarus* **66**, 341-358 (1986).

Novel types of ionic thermotropic liquid crystals

Duncan W. Bruce, David A. Dunmur, Elena Lalinde, Peter M. Maitlis & Peter Styring

Department of Chemistry, The University, Sheffield S3 7HF, UK

Liquid crystals are usually categorized as either lyotropic mesophases in which fluid anisotropy results from polar head-group packing of amphiphilic molecules, or as thermotropic mesophases where the orientational order arises from interactions between partially rigid anisotropic molecules. The phase types exhibited by lyotropic and thermotropic liquid crystals have distinctive structures, optical textures and physical properties. Inclusion of a rigid anisotropic moiety into a lyotropic liquid crystal gives an additional source of orientational ordering, and can lead to phase behaviour encompassing both thermotropic and lyotropic liquid crystals. We have prepared a series of silver-containing thermotropic liquid crystals based on the *bis*(stilbazole) silver (I) cation. Some members of this series, in association with the amphiphilic counter-ion lauryl sulphate, form liquid crystal mesophases characteristic of both lyotropic and thermotropic liquid crystals.

It has long been known¹⁻⁷ that anhydrous ionic amphiphiles can form liquid crystalline mesophases. The mesogenic species can be cationic such as alkylammonium⁷⁻¹⁵, or anionic such as alkyl carboxylates^{2,5,6,16-19} and the counter ions may be simple (Cl^- (refs 7, 10, 12-14), Br^- (ref. 15), Na^+ (refs 5, 6, 16-18) and Li^+ (ref. 19) or complex^{8,9,11,12,15}). The solid-phase behaviour of these systems is complicated, but they melt to a lamellar (neat) smectic-like structure, which clears to an apparently isotropic liquid at higher temperatures. Differential scanning calorimeter (DSC) studies^{6-8,10,12,18} show that the highest observed transition is weakly first order, characteristic of a mesophase/liquid transition, whereas the melting transition has a higher latent heat more typical of a crystal/mesophase transition. Luzzatti and Skoulios¹⁶ have examined the structure of

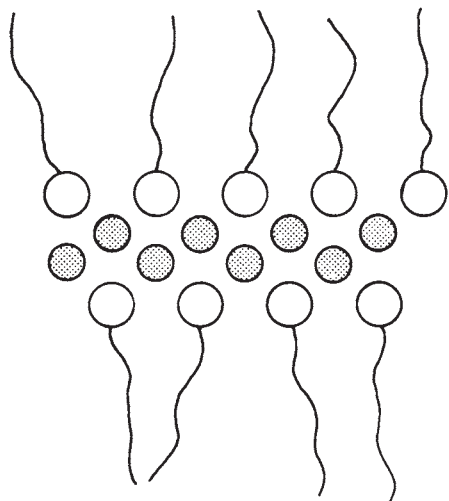


Fig. 1 Packing of amphiphilic ionic mesogens and spherical counter ions. In reality, the counter ions will be displaced above and below the plane of the figure to minimize repulsive interactions.

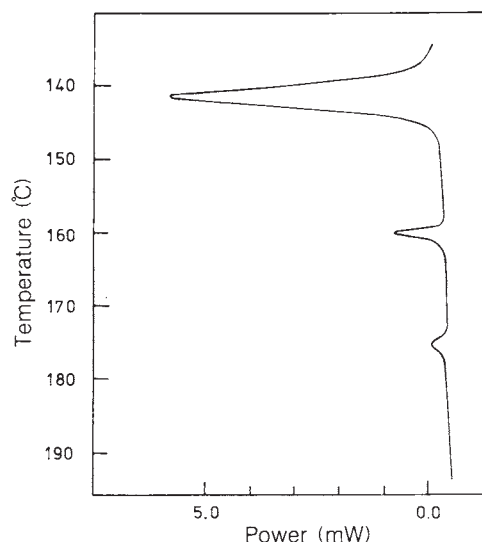
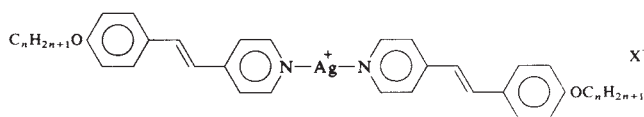


Fig. 2 DSC trace for $[\text{AgL}_2^{(8)}][\text{LS}]$.

these phases and suggested that they are based on aggregation of the polar head groups as indicated in Fig. 1. The counter ions are associated with the polar head groups, and the hydrocarbon chains fill space between them. In the lamellar (neat) phase, mobile bilayers are formed, but at lower temperatures more spatially ordered ribbon and disk phase structures may exist³. These are separated from the neat phase by a significant transitional enthalpy.

During our investigation of novel metal-containing liquid crystals^{20,21}, we discovered a new range of ionic mesogens in which the metal is complexed to an organic mesogenic ligand. The resulting cations will form ionic mesophases with either mesogenic or non-mesogenic anions. The former are of particular interest, as the complex metal cation has mesogenic properties similar to the parent (thermotropic) ligand, although the anion may be selected to be an ionic amphiphile. Mesophases formed in the ionic melt of these materials have characteristics of both thermotropic and amphiphilic phase types.

The silver-containing mesogenic cations we have prepared have the general structure:



The counterion X may be mesogenic (for example, lauryl sulphate LS) or non-mesogenic (such as BF_4^-). Reaction of AgBF_4 with substituted stilbazoles leads to complexes of the formula $[\text{AgL}_2^{(n)}][\text{BF}_4]$, where n is the number of carbon atoms in the substituted alkyl chain. The free stilbazole ligands are themselves mesogenic, and for $n > 5$ they show enantiotropic mesophase transitions. Coordination of the free ligands can enhance the stability of the mesophase: the silver ion links two stilbazole units and so increases the structural anisotropy and stabilizes the liquid crystal phase. Table 1 lists some properties of these new materials. The stilbazole ligands are synthesized by the method described by Heck²⁴. These are reacted with AgBF_4 in an organic solvent to give $[\text{AgL}_2^{(n)}][\text{BF}_4]$; further reaction with sodium lauryl sulphate yields $[\text{AgL}_2^{(n)}][\text{LS}]$. The complexes are light stable at room temperature, but become light and X-ray sensitive at high temperatures.

Figure 2 shows a typical DSC trace for a silver stilbazole complex ion with an amphiphilic counter ion. Two weak first-order transitions can be identified above the 'melting transition', and we have tentatively assigned the fluid phases as smectic B and lamellar. Complete identification of the phases must await X-ray and other studies. The existence of two mesophases above

Table 1 Properties of ionic *bis*(stilbazole) silver (I) mesogens

Compound	Transition		<i>T</i> (°C)	ΔH (Jg ⁻¹)	ΔS_m (R)
AgL ₂ ⁽³⁾ LS	K	S ₂	147	51.7	11.8
	S ₂	I	159	0.8	0.2
AgL ₂ ⁽⁴⁾ LS	K	S ₂	156	42.7	9.8
	S ₂	I	168	0.7	0.2
AgL ₂ ⁽⁸⁾ LS	K	S ₁	144	40.8	11.4
	S ₁	S ₂	164	3.6	0.9
	S ₂	I	177	1.4	0.4
AgL ₂ ⁽⁸⁾ BF ₄	K	M ₁	170	15.6	3.0
	M ₁	M ₂	270	16.4	2.5
	M ₂	I*	300	10.1	1.5

K, crystal; S, smectic (see text); M, unidentified mesophase; I, isotropic.

* With decomposition.

the 'melting' transition is in contrast with the normal behaviour of anhydrous ionic amphiphiles which show only one such phase, that is the lamellar or neat phase: other more ordered ribbon or disk phases occur below our designated melting transition. Figure 3 gives optical textures for the two high-temperature mesophases. The lower temperature texture is similar to the smectic B texture obtained for the free stilbazole ligands, whereas the higher temperature texture is more typical of a lamellar phase.

The mesophase structures of anhydrous ionic amphiphiles are based on the aggregation of polar head groups, where the charges are cancelled by counter ions (Fig. 1). Measurements of the order parameters of alkyl chain segments indicate²² that the orientational order is low compared with thermotropic liquid crystals. The new ionic liquid crystals reported here have anisotropic rigid cores that may be expected to enhance the

orientational order and hence the macroscopic anisotropy of the mesophases. At present, we have no information on the packing of anions and cations in the mesophases, but we might expect oppositely charged ions to interact in a manner similar to that shown in Fig. 1. The combination of thermotropic and amphiphilic phase properties in a single system has interesting consequences. For example the anisotropy of the rigid part of the mesogenic ions may ease the external control of macroscopic orientation by applied fields. In conventional Langmuir-Blodgett (LB) films, orientational order is achieved by surface packing of the polar head groups. By incorporating strongly anisotropic groups into the polar part of the molecule, it may be possible to produce orientationally ordered LB films more easily, and with larger macroscopic anisotropies. The effect of solvents on these mixed ionic liquid crystals has not been investigated, but may be expected to lead to a rich phase behaviour.

We are currently investigating mesophase formation between organic mesogenic anions and cations²³. The metal-containing cation in the materials described above may be replaced by an organic cation for example quaternary N-containing system, and these materials when combined with mesogenic anions also exhibit a rich mesophase behaviour.

We thank the SERC for financial support and for a research studentship to P.S.

Received 25 June; accepted 18 August 1986.

1. Vorlander, D. *Ber. dt. Chem. Ges.* **43**, 3120-3135 (1910).
2. Skoulios, A. & Luzzati, V. *Nature* **183**, 1310-1312 (1959).
3. Winsor, P. A. *Liquid Crystals and Plastic Crystals* (eds Gray, G. W. & Winsor, P. A.) 199 (Ellis Horwood, Chichester 1974).
4. Tiddy, G. J. T. *Phys. Rep.* **57**, 1-46 (1980).
5. Duruz, J. J. & Ubbelohde, A. R. *Proc. R. Soc. A* **330**, 1-13 (1972).
6. Busico, V., Ferraro, A. & Vacatello, M. *Molec. Cryst. Liq. Cryst.* **128**, 243-261 (1985).
7. Gault, J. D., Gallardo, H. A. & Muller, H. J. *Molec. Cryst. Liq. Cryst.* **130**, 163-177 (1985).
8. Landi, E. & Vacatello, M. *Thermochim. Acta* **12**, 141-146 (1975).
9. Landi, E., Salerno, V. & Vacatello, M. *Gazz. chim. ital.* **107**, 27-30 (1977).
10. Landi, E. & Vacatello, M. *Thermochim. Acta* **13**, 441-447 (1975).
11. Vacatello, M. & Busico, V. *Molec. Cryst. Liq. Cryst. Lett.* **64**, 127-132 (1981).
12. Busico, V., Castaldo, D. & Vacatello, M. *Molec. Cryst. Liq. Cryst.* **78**, 221-226 (1981).
13. Busico, V., Corradini, P. & Vacatello, M. *J. phys. Chem.* **86**, 1033-1034 (1982).
14. Busico, V., Cernicchiaro, P., Corradini, P. & Vacatello, M. *J. phys. Chem.* **87**, 1631-1635 (1983).
15. Paleos, C. M., Margomenou-Leonidopoulou, G. & Malliaris, A. *Chim. Chron., New Ser* **14**, 89-99 (1985).
16. Skoulios, A. E. & Luzzati, V. *Acta crystallogr.* **14**, 278-286 (1961).
17. Ubbelohde, A. R., Michels, H. J. & Duruz, J. J. *Nature* **228**, 50-52 (1970).
18. Madelmont, C. & Perron, R. *Bull. Soc. chim. Fr.* 1795-1798 (1974).
19. Busico, V., Ferraro, A. & Vacatello, M. *J. phys. Chem.* **88**, 4055-4058 (1984).
20. Bruce, D. W., Lalinde, E., Styring, P., Dunmur, D. A. & Maitlis, P. M. *JCS Chem. Commun.* 581-582 (1986).
21. UK Patent Appl. No. 8527377.
22. Tiddy, G. J. T. *Nuclear Mag. Reson. Spec. Per. Rep. chem. Soc.* **6**, 207-232 (1976).
23. Jokela, P. O., Jonsson, B. & Wennerstrom, H. *Prog. Coll. Polym. Sci.* **70**, 17-22 (1985).
24. Frank, W. C., Kim, Y. C. & Heck, R. F., *J. org. Chem.*, **43**, 2947 (1978).

Sulphur-induced faceting of platinum catalyst particles

P. J. F. Harris*

Department of Physical Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EP, UK

The poisoning of metal catalysts by sulphur is a serious problem in many chemical processes, but in some cases partial poisoning can produce beneficial changes in catalytic selectivity^{1,2}. The mechanism whereby adsorbed sulphur affects selectivity has been the subject of much debate; one view³ is that during poisoning a restructuring of the metal surface occurs that alters the activity of the catalyst towards structure-sensitive reactions. The adsorption of sulphur can induce 'faceting' of macroscopic metal surfaces (see for example ref. 4), so a similar phenomenon might occur when sulphur interacts with the small supported metal particles which are used in practical catalysts. Here I show, using transmission electron microscopy, that sulphur adsorption can produce a transformation in the morphology of alumina-supported

* Present address: TI Research, Hinxton, Saffron Walden, Essex CB10 1RH, UK.

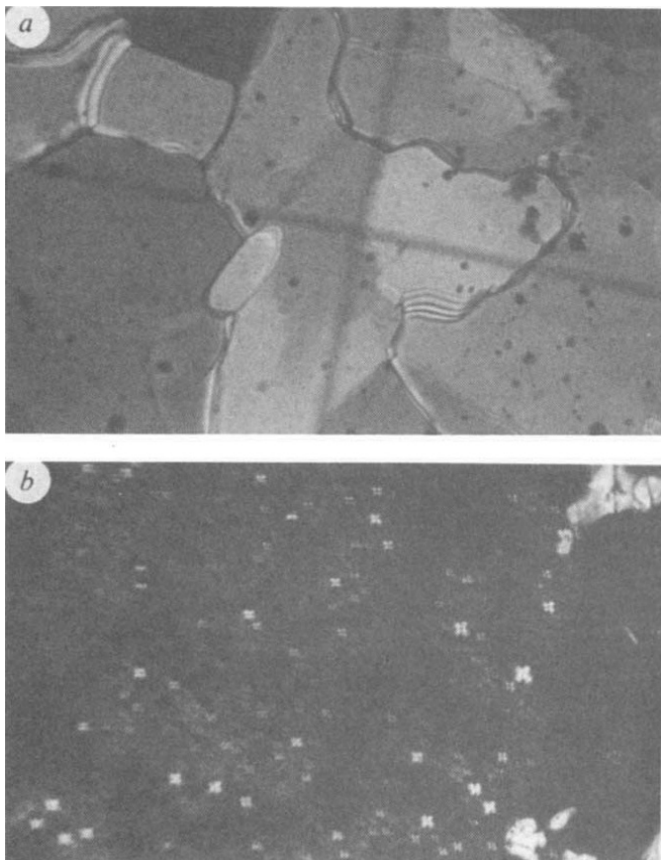


Fig. 3 Optical textures for two high-temperature phases of [AgL₂⁽⁸⁾][LS]. a, 148 °C: S, smectic B; b, 176 °C: S₂, lamellar.

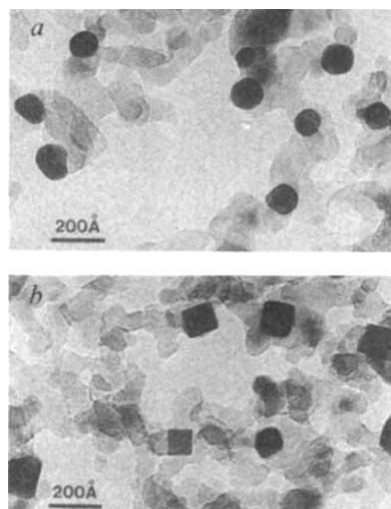


Fig. 1 Micrographs illustrating change in platinum particle morphology induced by sulphur adsorption. *a*, Specimen heated in air at 700 °C for 1 h. *b*, Specimen heated in flowing $\text{H}_2\text{S}/\text{H}_2$ at 500 °C for 16 h, following a similar air heat-treatment. Scale bar, 200 Å.

platinum particles, which apparently involves the formation of sharp (100) facets. This supports the restructuring model of sulphur poisoning³, and suggests that more attention should be paid to the role of adsorbate-induced faceting in the poisoning and promotion of supported metal catalysts.

The specimens used in this study were prepared by a novel technique which enables thin self-supporting catalyst films to be prepared directly on stainless-steel microscope grids. The technique, which is based on a process for applying catalytic coatings onto metallic or ceramic substrates, involved dipping the grids into an aqueous alumina sol to which a solution of tetrammine platinum (II) chloride had been added, drying the resultant film to a gel and then firing and reducing this to form thin sheets of platinum/alumina^{5,6}. Before carrying out the sulphur poisoning experiments, the freshly prepared specimens were heated in air at 700 °C for 1 h to increase the mean platinum particle size from 50 to 113 Å. This was necessary because the precise nature of sulphur-induced changes in particle morphology proved difficult to identify when fresh specimens were used. The air-sintered specimens were then placed in a controlled atmosphere furnace and a mixture containing 100 v.p.m. (volumes per million) hydrogen sulphide in hydrogen was passed through at a flow-rate of $30 \text{ cm}^3 \text{ min}^{-1}$. After flushing for 30 min at room temperature the specimens were heated to 500 °C over a period of 1 h, maintained at this temperature for 16 h and then allowed to cool. In these conditions, elemental sulphur would be expected to deposit onto the platinum particles, but formation of bulk sulphide would be unlikely^{1,2}. Specimens were examined using a JEOL 200CX transmission electron microscope operated in bright-field mode with an accelerating voltage of 200 kV.

Figure 1 shows micrographs of typical regions of unpoisoned and poisoned catalysts, while Fig. 2 shows the most commonly observed particle shapes before and after poisoning. Platinum particles in the unpoisoned catalysts were generally well rounded (Fig. 1*a*), with only slight faceting and no sharp edges or corners. Most particles were single crystals, and although many of these were irregular in shape, a significant proportion could be identified as rounded octahedra, indicating a degree of (111) faceting. Figure 2*a* shows a typical example of such a particle. The change in morphology induced by poisoning can be seen clearly in Fig. 1*b*. Instead of slight (111) faceting, single crystal particles in the poisoned catalyst exhibited predominantly square profiles strongly indicative of (100) faceting. Figure 2*b* shows another typical single crystal in a poisoned catalyst. The type of contrast

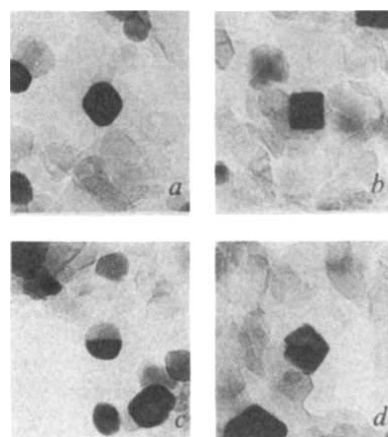


Fig. 2 Typical particle structures in unpoisoned and poisoned catalysts. *a*, Single crystal particle in air-heated specimen (rounded octahedron). *b*, Single crystal in sulphur-treated specimen. *c*, Twinned particle in air-heated specimen. *d*, Twinned particle in sulphur-treated specimen. Scale bar, 200 Å.

exhibited by these particles showed them to be three dimensional rather than 'raft-like' in form, indicating that their overall shapes were approximately cubic. In some cases, the (100) faceting was almost perfect with very little rounding of the corners visible, even at the highest magnification. The morphology of particles containing a single twin boundary was also transformed by sulphur adsorption. In the air-heated catalyst, such particles were often roughly circular in outline (Fig. 2*c*), while after poisoning they frequently exhibited the faceted profile shown in Fig. 2*d*. This profile is again indicative of (100) faceting and suggests an overall shape of the type sketched in Fig. 3.

To confirm that the observed changes in particle morphology were due to sulphur adsorption, a control experiment was carried out which involved heating catalyst specimens in pure hydrogen in conditions identical to those used in the hydrogen sulphide/hydrogen treatments. This resulted in relatively little change in particle shape, but the degree of faceting appeared to decrease slightly producing particles with almost circular profiles.

The observations reported here are consistent with studies of macroscopic metal surfaces. For example, Schmidt and Luss⁴ have shown that treatment of platinum/rhodium gauzes with hydrogen sulphide results in the formation of (100) surface planes, while McCarroll *et al.*⁷ report a "reorientation" of the (111) face of nickel to (100) in the presence of hydrogen sulphide. However, sulphur-induced faceting has not previously been observed directly using specimens which closely resemble real supported catalysts. Indeed, the effect of adsorption on the surface structure of very small metal particles has received surprisingly little attention. *In situ* transmission electron microscopy work⁸⁻¹⁰ has indicated that the shape of very small noble metal particles can be changed by exposure to oxygen, carbon monoxide and other gases, although these results were partly

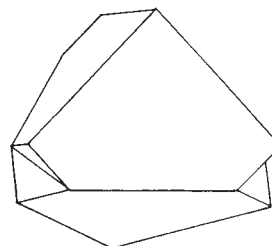


Fig. 3 Shape of twinned particle bounded by (100) facets.

explained in terms of changes in the particle-support interaction. Gillet *et al.*^{11,12} have reported changes in the morphology of palladium particles induced by carbon monoxide/oxygen mixtures, while Wang *et al.*¹³ have found that platinum particles develop (100) facets when heated in hydrogen, in contrast to the findings of this study. Previous work¹⁴ has shown that heating platinum/alumina specimens in a vacuum furnace induces the formation of strong, mainly (111), facets. This was attributed to the deposition of carbon onto the surfaces of the particles during heat treatment in the relatively poor vacuum. Lamber and Romanowski^{15,16} have made a similar observation but again they interpret their results in terms of a metal-support interaction. Further studies of the type described here are required if a more fundamental understanding of the factors which control the structure and activity of supported metal catalysts is to be gained.

I thank Professor J. M. Thomas for discussions, the SERC for a postdoctoral fellowship and AERE Harwell for provision of materials.

Received 7 July; accepted 18 August 1986.

1. Oudar, J. *Catal. Rev.—Sci. Engng* **22**, 171–195 (1980).
2. Bartholomew, C. H., Agrawal, P. K. & Katzer, J. R. *Adv. Catal.* **31**, 135–242 (1982).
3. Somorjai, G. A. *J. Catal.* **27**, 453–456 (1972).
4. Schmidt, L. D. & Luss, D. J. *Catal.* **22**, 269–279 (1971).
5. Harris, P. J. F., Boyes, E. D. & Cairns, J. A. *J. Catal.* **82**, 127–146 (1983).
6. Harris, P. J. F. *J. Catal.* **97**, 527–542 (1986).
7. McCarroll, J. J., Edmonds, T. & Pitkethly, R. C. *Nature* **223**, 1260–1262 (1969).
8. Heinemann, K., Osaka, T. & Poppa, H. *Ultramicroscopy* **12**, 9–18 (1983).
9. Heinemann, K., Osaka, T., Poppa, H. & Avalos-Borja, M. *J. Catal.* **83**, 61–78 (1983).
10. Park, C., Durrer, W. G., Poppa, H. & Dickinson, J. T. *J. Catal.* **95**, 361–369 (1985).
11. Gillet, E., Channakhone, S., Matolin, V. & Gillet, M. *Surf. Sci.* **152/153**, 603–614 (1985).
12. Gillet, M. F. & Channakhone, S. *J. Catal.* **97**, 427–436 (1986).
13. Wang, T., Lee, C. & Schmidt, L. D. *Surf. Sci.* **163**, 181–197 (1985).
14. Harris, P. J. F. *Appl. Catal.* **16**, 439–442 (1985).
15. Romanowski, W. & Lamber, R. *Thin Solid Films* **127**, 139–157 (1985).
16. Lamber, R. *Thin Solid Films* **128**, L29–L32 (1985).

Role of plutonism in low-pressure metamorphic belt formation

D. R. Lux, J. J. DeYoreo, C. V. Guldotti
& E. R. Decker

Department of Geological Sciences, University of Maine, Orono, Maine 04469, USA

Wickham and Oxburgh¹ recently proposed that low-pressure/high-temperature (low-*P*/high-*T*) metamorphism in the eastern Pyrenees, and possibly all low-*P*/high-*T* metamorphic belts, resulted from anomalously high mantle heat flow brought about by rifting. Their model is largely constrained by the presence of nearby synmetamorphic rift-related sedimentary rocks and the interpretation that the migmatites and granites are the product of *in situ* melting in the presence of an anomalously steep geotherm. Here we present an alternative model, in which low-*P*/high-*T* metamorphism (pro-grade reactions at pressures near or below the Al_2SiO_5 triple point) results from contact effects near sill-like igneous intrusions at intermediate crustal levels. Low-*P*/high-*T* conditions can be achieved through this process in regions of continent–continent collision with normal mantle heat flux as well as in zones of extension. Our model is based on studies of the low-*P*/high-*T* metamorphic terrane in the New England Appalachians.

Regional metamorphism in zones of continent–continent collision can be modelled mathematically as a thermal consequence of large-scale crustal thickening and subsequent erosion^{2–5}. Such models depend on a previous knowledge of the burial and erosion history of the region. In addition, it is assumed that crustal radioactivity and a constant mantle heat flux are the only heat sources and that conduction is the only mode of heat

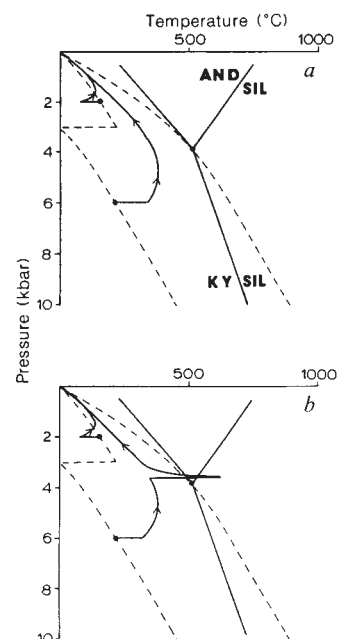


Fig. 1 Hypothetical *P*-*T*-*t* paths after instantaneous thrusting of a 12-km slab, projected onto the pressure-temperature plane. The dashed line to the left is the temperature distribution immediately after thrusting; that to the right is the equilibrium temperature distribution that would result if no erosion was to occur. Solid curves give the time evolution of the temperature for crustal horizons in the upper and lower plates when a period of quiescence proceeds erosion and *a*, magmatic intrusions are ignored, *b*, a magmatic intrusion in the lower plate is considered. Mantle heat flux is 37 mW m^{-2} . Radioactive heat production of 4.2 mW m^{-3} is uniformly distributed throughout the upper plate and the top 7 km of the lower plate. The aluminosilicate triple point is after ref. 35.

transfer. Although the pressure-temperature-time (*P*-*T*-*t*) path followed by a particular crustal horizon is a function of many parameters, two important generalizations can be made. (1) Melting in the lower crust can be a consequence of crustal thickening. (2) *P*-*T*-*t* trajectories only pass through both the sillimanite (SIL) and the andalusite (AND) stability fields in models with extreme values of the thermal conductivity, mantle heat flux or radioactive heat production in the crust. Moreover, they do so with retrograde metamorphic conditions. Prograde AND to SIL reactions are not predicted (see Fig. 1*a*).

For Barrovian metamorphic terranes, these mathematical models yield results that are consistent with the geological observations^{2,3,5}. However, they fail to predict the prograde AND to SIL metamorphism that is characteristic of many low-*P*/high-*T* belts^{6,7}. Hence, conduction in a tectonically thickened crust with a constant mantle heat flux does not account for the main feature of many low-pressure metamorphic belts. Therefore, we suggest that granitoid magmas produced in the lower crust in response to crustal thickening may be emplaced at intermediate levels, causing deep-level contact effects with low-*P*/high-*T* mineral assemblages. Such crustally-derived plutons are abundant in many low-*P*/high-*T* belts^{6,7}. Qualitatively, the thermal effect of a magmatic intrusion is to increase rapidly and isobarically the temperature of the adjacent country rock (Fig. 1*b*). As will be shown below, in regions of the thickened crust, prograde AND to SIL metamorphism can be produced by this mechanism assuming normal mantle heat flux. Wickham and Oxburgh¹ proposed a different mechanism. They suggested that anomalously high mantle heat flux and convection through mafic magmas from the mantle initiated by lithospheric extension would lead to the formation of low-*P*/high-*T* assemblages. The granitoid plutons which are abundant in many low-*P*/high-*T* belts are interpreted by them to be a result of *in situ* melting.

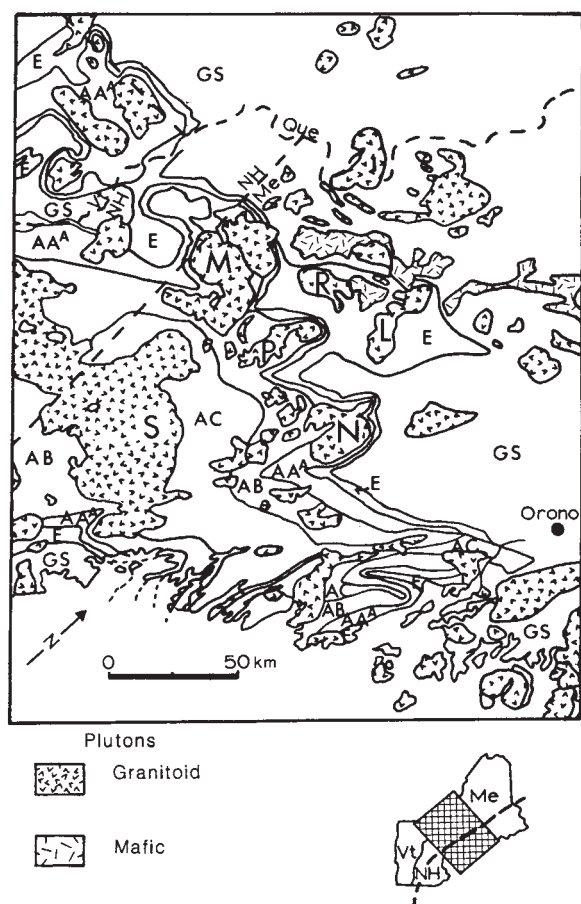


Fig. 2 Metamorphic map after ref. 9 of the northern end of the New England metamorphic high. Metamorphic facies designation: GS, greenschist characterized by chlorite + actinolite or biotite; E, epidote amphibolite characterized by Fe-garnet in pelites or Na-plagioclase + epidote + hornblende in basic rocks; AA, lower amphibolite characterized by staurolite and kyanite (AA^k) or andalusite (AA^a) in pelites and diopside in basic rocks; AB, medium amphibolite characterized by sillimanite in pelites; AC, upper amphibolite characterized by sillimanite + K-feldspar in pelites. S, Sebago batholith; P, Phillips pluton; M, Mooselookmeguntic batholith; R, Reddington pluton; N, Norridgework pluton; L, Lexington pluton. The axis of the Merrimack Synclinorium is depicted by the dashed line on the index map.

A further consequence of their model is that the entire crust below the zone of low- P /high- T metamorphism is raised above the wet granite solidus.

The northern end of the New England high-grade terrane is in many ways typical of low- P /high- T metamorphic belts. Peraluminous granites are abundant as is prograde AND to SIL metamorphism^{8,9}. Although the highest grade of metamorphism tends to occur near the plutons, distinct contact aureoles are usually lacking, and high-grade metamorphism can occur tens of kilometres away from the nearest outcrop of granite, suggesting that the granites are an unlikely heat source for the metamorphism. In spite of this, the following geochronological and petrological observations lead us to conclude that at least in northern New England, and perhaps in many low- P /high- T zones, the plutons were the immediate source of heat for the metamorphism.

The high-grade terrane terminates in a series of north-east projecting lobes (Fig. 2). The outcrop patterns of the granites and the isograds of these lobes show a strong spatial correlation¹⁰. Moreover, in some cases the isograds of one lobe are overprinted by those of another^{8,9}, indicating that the lobes were produced by temporally separate events.

A minimum of three temporally distinct metamorphic events separated by periods of cooling are recognized in western Maine on a regional scale^{8,9}. All clearly postdate the early Devonian (Acadian) deformation of the region and seem to coincide with periods of igneous activity. The first two metamorphisms are Devonian in age^{8,9} and the third is Carboniferous in age^{11,12}.

There is widespread occurrence of equilibrium or near-equilibrium mineral assemblages that can be explained by simple prograde reactions^{8,9}. The pressures and temperatures required to produce these reactions imply P - T arrays with gradients of 80–100 °C km⁻¹ over short distances at depths of 10–15 km with maximum temperatures of 450–650 °C (refs 13, 14). We believe that this thermal structure could have been produced only by local, transient heat sources.

⁴⁰Ar/³⁹Ar dating suggests that hornblendes from the southern half of the Mooselookmeguntic batholith were reset ~325 Myr ago¹¹. This is also the crystallization age of the nearby Sebago batholith as determined from both Rb/Sr whole rock and U-Pb zircon dating^{12,15}.

North of the Sebago batholith, both pressures and temperatures of metamorphism increase towards the batholith. In contrast, isograds south of the Sebago are inverted, with pressure increasing away from the batholith and temperatures increasing towards it¹⁴.

⁴⁰Ar/³⁹Ar release spectrum ages for hornblende from the northern part of the Mooselookmeguntic pluton are identical to the crystallization age of the pluton as determined by the Rb/Sr whole rock isochron technique¹¹. Cooling to ambient temperature following pluton emplacement was rapid and the ambient temperature was below the closure temperature for Ar loss in hornblende, ~500–550 °C (ref. 16). Clearly, the pluton did not form by *in situ* melting but was emplaced from below. We conclude from these observations that the plutons are the primary feature and the adjacent metamorphism is secondary.

Although they produce only short-range thermal effects¹⁷, we contend that plutons are the heat source for the thousands of square kilometres of low- P /high- T metamorphism of western Maine. This paradox is resolved by considering the geometry of the plutons of the high-grade metamorphic terrane. Gravity studies indicate that they are 1–2-km-thick sill-like plutons that dip to the north-east at 4–6° (refs 18–20). Many extend far beyond their surface exposures^{18–20}. Consequently, areas far from a surface contact may be only a few kilometres above that contact at depth.

For example, although the surface exposures of the Mooselookmeguntic batholith and the Reddington pluton are separated by 10–15 km, gravity studies across the region suggest that the Mooselookmeguntic batholith dips towards the Reddington pluton and extends beneath it, staying within 1 km of the surface²⁰. Hence, as shown below, it is a probable heat source for the staurolite to sillimanite grade metamorphism of the intervening region.

We have examined the thermal history of the region to answer two questions. First, are the plutons likely to be a consequence of conductive heating subsequent to crustal thickening? Second, do the thin tabular plutons contain sufficient heat to produce high-grade metamorphism?

Most plutons that occur in the Merrimack Synclinorium are undeformed and have ages that cluster around 400 Myr and 370 Myr (ref. 21). In western Maine, the Acadian burial history of the Merrimack Synclinorium is one of sedimentation followed by folding and thrusting. A total of 10–14 km of strata were deposited between late Ordovician and early Devonian time (440–400 Myr)²². Just before 400 Myr ago, the pile was tectonically thickened as a result of continent–continent collision^{23–25}. The seismically determined thickness of metasedimentary section in the present day Merrimack Synclinorium in central Maine is 10–12 km (ref. 26), and the present erosion surface was at a depth equivalent to 3–4 kbar shortly after 400 Myr (refs 7, 8). Thus the 400-Myr deformational event led to 10–15 km of

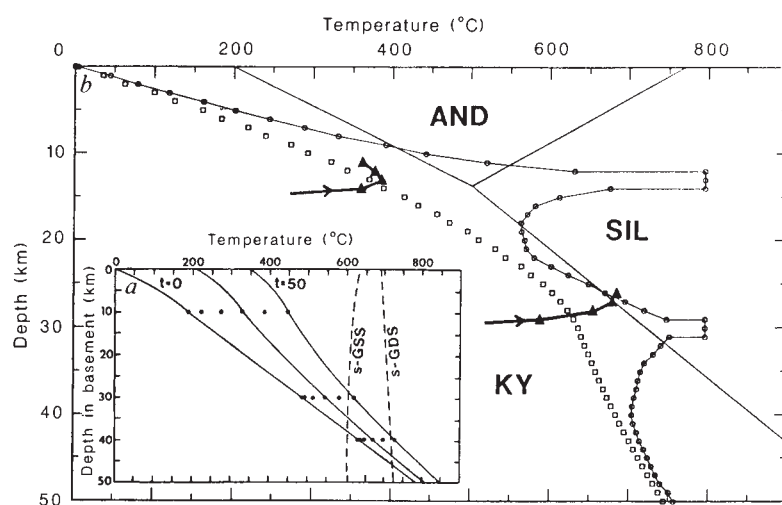


Fig. 3 Temperature-time history for western Maine. *a*, Effect of sedimentation on basement temperatures. Initial temperature distribution, ($t=0$) based on present day heat-flow-heat-production measurements^{28,36}. The volumetric heat production, the thermal conductivity and the thermal diffusivity of the sediments are 2 mW m^{-3} , $1.8 \text{ W m}^{-1} \text{ K}^{-1}$, and $32 \text{ km}^2 \text{ Myr}^{-1}$ respectively. The deposition rate is 0.3 km Myr^{-1} . Each circle represents a 10-Myr step. Saturated (s-GSS) and dry (s-GDS) solidus curves for S-type granite after ref. 29. *b*, Temperature distribution after homogeneous doubling of a 12.6-km pile of sediments (deposited over 42 Myr). $\square$, 30 Myr after thickening. $\circ$, 30 Myr after thickening with 2-km-thick igneous sheets centred at 13 and 30 km. $\blacktriangle$, P - T - t paths of horizons initially at 15 and 30 km depth immediately after thickening. Each triangle represents 20 Myr. The erosion rate is 0.05 km Myr^{-1} and the conductivity and diffusivity are $2.5 \text{ W m}^{-1} \text{ K}^{-1}$ and $32 \text{ km}^2 \text{ Myr}^{-1}$ respectively and are uniform with depth. (Temperature profiles exceeding the saturated solidus would be modified due to melting.) The aluminosilicate triple point is after ref. 35.

thickening. Petrological and isotopic age studies show that little if any erosion occurred between 400 and 370 Myr (refs 8, 9, 11) and that the erosion rate from 370 to 280 Myr was $\sim 0.05 \text{ km Myr}^{-1}$.

The thermal consequences of this burial history have been modelled numerically using a Crank-Nicolson²⁷ implicit finite difference method. The results, in agreement with calculations by Birch *et al.*²⁸, suggest that 10–15 km of sedimentation at a uniform rate of 0.3 km Myr^{-1} for 30–50 Myr could produce granitic melts in the lower crust in basement terranes that are characterized by normal mantle and surface heat flow (see inset, Fig. 3). This suggests that the sedimentation in the Merrimack Synclinorium may have led to the 400 Myr plutonism. Also shown in Fig. 3 are the results of deposition of a 12.6-km pile of sediments deposited over 42 Myr followed by homogeneous doubling of the thickness of the pile. Within 30 Myr of thickening, nearly all the basement is raised above the saturated solidus for S-type granites²⁹ making renewed igneous activity by 370 Myr probable. (Temperature profiles exceeding the saturated solidus would be modified due to melting.)

The effect of thin tabular plutons can now be estimated. Figure 3 shows the results of models that assume 2-km thick, 800°C (refs 30, 31) magmatic sheets emplaced at 13 km and at 30 km. The effect is much smaller at the deeper level and, as the P - T - t paths show, the deep horizon would attain equally high temperatures through conductive heating in the absence of plutonism. At the more shallow level, the effect is to produce prograde AND to SIL metamorphism with maximum temperatures of $\sim 650^\circ\text{C}$ with gradients in the P - T arrays of 80 – $100^\circ\text{C km}^{-1}$. Medium- to high-grade metamorphism is predicted only out to $\sim 2 \text{ km}$ from the sheet. For a sufficiently extensive sheet dipping at 4 – 6° , areas up to $\sim 25 \text{ km}$ from the surface contact would be within this high-grade metamorphic aureole.

Note that both prograde AND to SIL and KY (kyanite) to SIL metamorphism are predicted. A transition from the former to the latter should be observed as the depth of emplacement of the plutons increases. This occurred in western Maine at the level of intrusion of the Sebago batholith. AND to SIL metamorphism is observed at the northern edge⁸, whereas KY to SIL reactions occurred at the southern edge¹⁴.

If the total amount of burial is much less than estimated above, and if the volumetric heat production of the basement and the sediments was anomalously low, or if a sufficient volume of granitic magma ($\sim 800^\circ\text{C}$) cannot be produced, then high transient mantle heat flux may be required to account for the plutonism.

The similarity of the New England low- P /high- T terrane to other metamorphic belts raises the question of whether granitic

intrusions are often the source of prograde AND to SIL metamorphism in low- P /high- T belts¹¹. Because widespread occurrence of low-pressure, high-grade rocks in New England is highly dependent on the geometry of the granitic intrusions, the answer must be based on an understanding of the mechanism that governs the emplacement of granites. The results of recent models suggest that silicic magmas generated in the lower crust will rise diapirically as long as the surrounding rocks can deform plastically³². Depending on the geothermal gradient, this transition from brittle to ductile behaviour occurs at a depth of 10–20 km (ref. 33) where the diapirs spread laterally and form tabular plutons³⁴. This is a typical depth range for low- P /high- T metamorphism^{6,7}. The source of heat for the plutonism may not be the same in each belt. They can be a result of crustal thickening during collision, high mantle flux during extension, or melting point depression due to the release of volatiles from a subducted plate. Regardless of the source, we conclude that plutons may control the metamorphism in many low- P /high- T belts, and that they do so because of their geometry, which is a natural consequence of the rheological properties of rock.

Supported by NSF grants EAR-82-06549 and ISP-8011448.

Received 27 March; accepted 19 August 1986.

- Wickham, M. W. & Oxburgh, E. R. *Nature* **318**, 330–333 (1985).
- England, P. C. & Thompson, A. B. *J. Petrol.* **25**, 894–928 (1984).
- Royden, L. & Hodges, K. V. *J. geophys. Res.* **89**, 7091–7106 (1984).
- England, P. C. & Richardson, S. W. *J. geol. Soc. Lond.* **134**, 201–213 (1977).
- Oxburgh, E. R. & Turcotte, D. L. *Schweiz. miner. petrogr. Mitt.* **54**, 641–662 (1974).
- Miyashiro, A. *Petrology* **2**, 277–311 (1961); *Tectonophysics* **17**, 241–254 (1973); *Metamorphism and Metamorphic Belts* (Wiley, New York, 1973).
- Zwart, H. J. *Geologie Mijnb.* **46**, 283–309 (1967).
- Holdaway, M. J., Guidotti, C. V., Novak, J. M. & Nenry, W. E. *Bull. geol. Soc. Am.* **93**, 572–584 (1982).
- Guidotti, C. V., Trzcinski, W. E. & Holdaway, M. J. in *Regional Trends in the Geology of the Appalachian-Caledonian-Hercynian-Mauritanide Orogen* 235–247 (ed. Schenk, P. E.) (Reidel, Dordrecht, 1983).
- Thompson, J. B. & Norton, S. A. in *Studies of Appalachian Geology* (eds E-an Zen *et al.*) 319–327 (Wiley, New York, 1968).
- Lux, D. R. & Guidotti, C. V. *Geology* **13**, 696–700 (1985).
- Aleinikoff, J. N., Moench, R. H. & Lyons, J. B. *Bull. geol. Soc. Am.* **96**, 990–996 (1985).
- Cheney, J. T. & Guidotti, C. V. *Am. J. Sci.* **279**, 411–434 (1979).
- Thomson, J. A. thesis, Univ. Maine at Orono (1985).
- Hayward, J. A. & Gaudette, H. E. *Geol. Soc. Am. Abstr. Prog.* **16**, 22 (1984).
- Harrison, T. M. *Contr. Miner. Petrol.* **78**, 324–331 (1981).
- Jaeger, J. C. *Rev. Geophys.* **2**, 443–466 (1964).
- Hodge, D. S. *et al. Am. J. Sci.* **282**, 1289–1324 (1982).
- Nielson, D. L., Clark, R. G., Lyons, J. B., England, E. J. & Borns, D. J. *Geol. Soc. Am. Mem.* **146**, 301–318 (1976).
- Carnese, M. J. thesis, Univ. New Hampshire (1983).
- Osberg, P. H. in *Regional Trends in the Geology of the Appalachian-Caledonian-Hercynian-Mauritanide Orogen* (ed. Schenk, P. E.) 315–337 (Reidel, Dordrecht 1983).
- Moench, R. H. & Zartman, R. E. *Geol. Soc. Am. Mem.* **146**, 203–238 (1976).
- Osberg, P. H. *Geol. Surv. Can. Pap.* **78-13**, 137–147 (1978).
- Zen, E-an *Geol. Soc. Am. Mem.* **158**, 55–81 (1983).
- Bradley, D. C. *J. Geol.* **91**, 381–400 (1983).
- Stewart, D. B., Luetgert, J. H., Unger, J. D. & Phillips, J. D. *Geol. Soc. Am. Astr.* **17**, 727 (1985).
- Smith, G. D. *Numerical Solution of Partial Differential Equations* (Oxford University Press, 1978).

28. Birch, F., Roy, R. F. & Decker, E. R. in *Studies of Appalachian Geology* (ed. E-an Zen) 437-451 (Interscience, New York, 1968).
29. Huang, W. L. & Wyllie, P. J. *Contr. Miner. Petrol.* **42**, 1-14 (1973).
30. Clemens, J. D. & Wall, V. J. *Can. Miner.* **19**, 111-132 (1981).
31. Tewhey, thesis, Brown University (1975).
32. Marsh, B. D. *Am. J. Sci.* **282**, 808 (1982).
33. Eaton, G. P. A. *Rev. Earth planet. Sci.* **10**, 409-440 (1982).
34. Whitney, J. A. & Stormer, J. C. *Science* **231**, 483-486 (1986).
35. Holdaway, M. J. *Am. J. Sci.* **271**, 97-131 (1971).
36. Jaupart, C., Mann, J. R. & Simmons, G. *Earth planet. Sci. Lett.* **59**, 267-287 (1982).

Anoxic hypolimnion is a significant source of biogenic toluene

F. Jüttner & J. J. Henatsch

Institut für Chemische Pflanzenphysiologie der Universität Tübingen, Corrensstr.41, D-74 Tübingen, FRG

Toluene is the predominant aromatic compound in freshwater lakes in Central Europe¹⁻³ and sea water⁴⁻⁶. Its concentrations exceed those of the xylene and ethyltoluene isomers both of which also occur in a comparable concentration range. Toluene is also the most important aromatic compound in the air of Europe⁷, North America⁸ and Australia⁹. Anthropogenic sources are held to be responsible for the high concentrations of toluene in water and air. Significant amounts have been introduced by oil spillage, emissions of petrol and diesel fuelled vehicles and other combustion processes^{10,11}. Toluene is therefore often used as a measure of pollution. We report here that while investigating the VOC (volatile organic compounds) in a stratified lake, we found that appreciable amounts of toluene can also be biogenically produced in the anoxic hypolimnion.

Water samples were taken from different depths of a small eutrophic lake (Schleinssee, south-west Germany; maximum depth, 10.5 m; area, 0.15 km²) at 3-weekly intervals for a period of 1 year and the VOC were qualitatively and quantitatively analysed by capillary gas chromatography and mass spectrometry¹². For this purpose 10-l samples were taken at 1 m intervals from depths between 1 and 10 m by pumping water up through glass tubes from different lengths into glass bottles. From this water the VOC were stripped in a closed system and sorbed on a Tenax TA trap. After thermal desorption, toluene and the other VOC were separated by gas chromatography on a glass capillary column (38 m, UCON 50 HB 5100, H₂ carrier gas, 4 min at 0 °C, then temperature was increased by 5 °C min⁻¹ up to 180 °C). The identity of compounds was confirmed by mass spectrometry. The mean error of the quantitative determination of toluene was $\pm 12\%$.

Fig. 2 Distribution of volatile organic substances (that is toluene, *m*-xylene, octan-3-ol, geosmin, *p*-cresol) in a depth profile in the Schleinssee on 22 November 1984. The staggered lines represent the flame-ionization signals of the VOC obtained from water from different depths. The numbers give the absolute concentrations of the VOC in ng l⁻¹. The 11-m sample was a mixture between soft sediment, interstitial water and water close to the sediment. Also shown is the distribution of the concentrations of oxygen, nitrate, ammonia and sulphide in the depth profile.

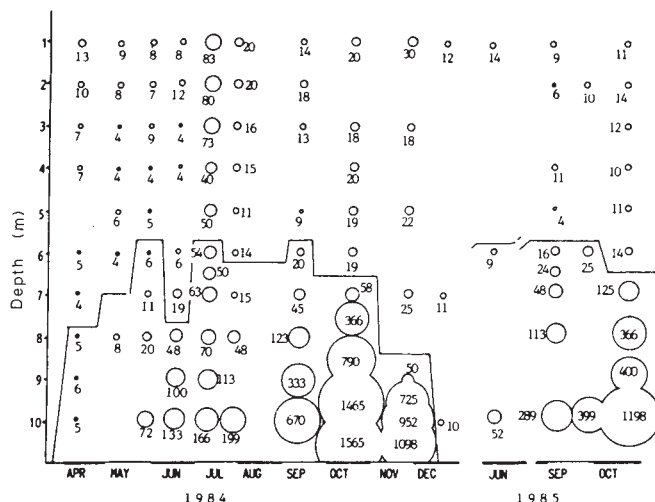
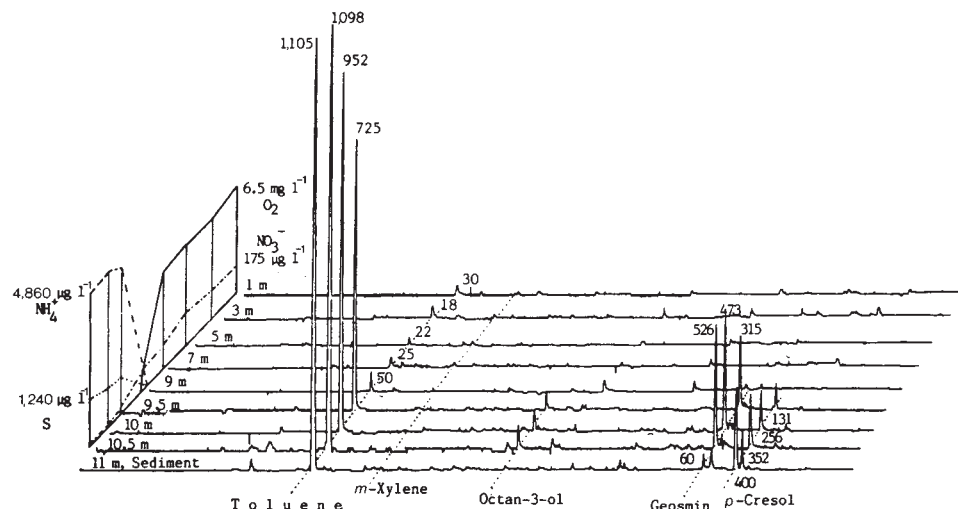


Fig. 1 Seasonal depth profiles of the concentrations of toluene (areas of circles). The numbers beside or within the circles represent the toluene concentrations (ng l⁻¹). The stepped horizontal line indicates the upper boundary of the anoxic part in the Schleinssee 1984 and 1985.

During the spring turnover the Schleinssee water contained oxygen throughout the depth profile. Toluene was present in low concentrations not exceeding 10-20 ng l⁻¹, a range typical of clean waters in the region. After stratification and formation of an anoxic hypolimnion, the toluene concentrations in the water body did not initially change. With the onset of summer a steady increase of toluene concentrations in the anoxic hypolimnion was observed while those of the epilimnion remained nearly constant. In the autumn, the depth profiles were characterized by only small variations in the toluene concentration in the epilimnion but a dramatic increase of toluene concentration in the anoxic metalimnion and a further steady increase, which continued to the sediment, in the hypolimnion. Extraordinarily high toluene concentrations (1.5 µg l⁻¹ 1 m above the sediment) were observed in the autumn after the anoxic hypolimnion had developed to its greatest extent (Fig. 1). At this depth, concentrations of ammonia were also at their highest (Fig. 2). The total amount of toluene in the lake increased from 10 g during the anaerobic conditions of the spring turnover to 150 g in October (Fig. 3) when an anoxic hypolimnion had developed. To determine the possible cause of the toluene increase the annual cycle was studied for a second year in 1985 and showed similar seasonal fluctuations in the toluene concentration (Figs 1 and 3). The reproducible occurrence of this

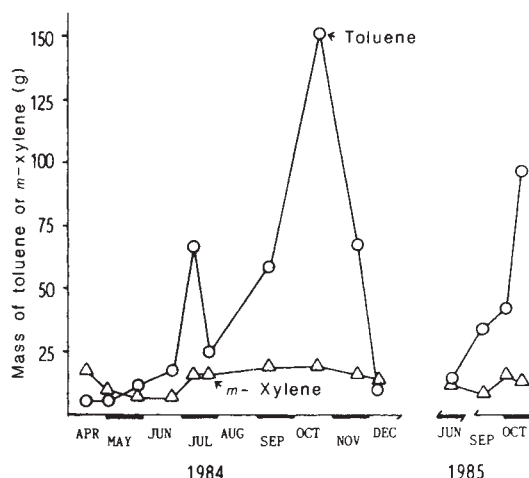


Fig. 3 Total amount of toluene and *m*-xylene in the water body of the Schleinsee during 1984 and 1985.

phenomenon is a strong indication of a biogenic source of toluene formation. If toluene had been introduced through contamination by oil or exhaust products of combustion processes then an increase of other aromatic compounds such as xylenes, ethylbenzene and ethyltoluenes would have been observed. The analysis of these compounds, however, exhibited essentially no change during the year. The levels observed represent those usually found in weakly polluted waters.

Investigations performed in the Gnadensee, a eutrophied part of Lake Constance that developed an anoxic hypolimnion in 1983, indicated that an increase in toluene concentration in the anoxic hypolimnion (five times greater than in the oxygenated epilimnion) also occurred. Thus, formation of toluene seems to be a widespread phenomenon in anoxic hypolimnions of eutrophic lakes. Increased toluene concentrations in sediment near layers of oxygenated hypolimnions have also been reported^{2,13}. However, there has been no explanation of this phenomenon.

A biogenic source of toluene which could account for the amounts reported here has been reported by Pons *et al.*¹⁴. They demonstrated a high incorporation of deuterium from deuterated phenylalanine into toluene with cultures of *Clostridium aerofotidum*. The high rate of this conversion makes it plausible that such a reaction could be the source of toluene in the hypolimnion. Assuming that the fraction of phenylalanine in the protein¹⁵ in the hypolimnion is 4.8%, then 8.4 kg of phenylalanine could have been degraded between September and October. The rapid rise in and extremely high concentrations of ammonia during autumn, 2,160 $\mu\text{g l}^{-1}$ in September, 5,760 $\mu\text{g l}^{-1}$ in October, provides additional, albeit circumstantial, evidence for intense protein metabolism in the anoxic hypolimnion.

The assumption that phenylalanine may be an essential precursor of the toluene is supported by the associated occurrence of *p*-cresol which was found exclusively in the anoxic hypolimnion (Fig. 2). *p*-Cresol can be formed in a similar degradation reaction with hydroxyphenylalanine that has been described for clostridia¹⁶ and *Lactobacillus*¹⁷.

An alternative biogenic source of toluene is carotene¹⁸, but quantitative considerations make it unlikely that this was the primary source of the toluene accumulation reported here. Using isolates of anaerobes from sea sediments it was demonstrated that toluene formed in metal cans when β -carotene was added to the cultures as substrate. However, the total transformation was rather low yielding only 35 mg of toluene per kg β -carotene. Thus, this source of toluene cannot be a major metabolic pathway. One can compare these results with the situation in the Schleinsee between September and October: although 90 g of

toluene were formed, only 18.7 kg of carotenoids were degraded in the hypolimnion if the input of organic matter from the epilimnion by sedimentation is neglected. The breakdown of the stratification in autumn that leads to oxygenated conditions in the lake does not result in a general initial increase of toluene concentrations in the lake. In oxygenated conditions, the degradation of toluene appears to be too fast to allow any accumulation of toluene. Thus, the low concentration $\sim 11 \text{ ng l}^{-1}$ were re-established after termination of the stratification. Intense degradation of toluene has also been observed in sea water contaminated by ballast water from oil tankers¹⁹. The ability of microorganisms to degrade toluene resides in genes coded for by a plasmid²⁰. This observation is a further indication that toluene is a widely occurring natural compound whose accumulation can be expected in many anoxic systems. Only the enormous difficulty in differentiating between biogenic and anthropogenic sources of toluene has hindered the identification of the biogenic sources.

This study was supported by the Deutsche Forschungsgemeinschaft.

Received 28 May 1986; accepted 21 August 1986.

- Jüttner, F., Höflacher, B. & Wurster, K. J. *Phycol.* **22**, 169-175 (1986).
- Internationale Gewässerschutzkommission für den Bodensee Rep. no. 32 (1985).
- Jüttner, F. *Wat. Sci. Technol.* **15**, 247-257 (1983).
- Gschwend, P. M., Zafriou, O. C., Mantoura, R. F. C., Schwarzenbach, R. P. & Gagosian, R. B. *Envir. Sci. Technol.* **16**, 31-38 (1982).
- Sauer, Jr., T. C. *Limnol. Oceanogr.* **25**, 338-351 (1980).
- Gschwend, P., Zafriou, O. C. & Gagosian, R. B. *Limnol. Oceanogr.* **25**, 1044-1053 (1980).
- Bruckmann, P., Beier, R. & Krautscheid, S. *Staub-Reinhalt. Luft* **43**, 404-410 (1983).
- Lonnemann, W. A., Sella, R. L. & Bufalini, J. J. *Envir. Sci. Technol.* **12**, 459-463 (1978).
- Nelson, P. F. & Quigley, S. M. *Envir. Sci. Technol.* **16**, 650-654 (1982).
- Nelson, P. F. & Quigley, S. M. *Atmos. Envir.* **18**, 79-87 (1984).
- Dulson, W. *Schr. Ver. Wasser, Boden Luft Hyg.* **47**, 5-128 (1978).
- Jüttner, F. *Appl. Envir. Microbiol.* **47**, 814-820 (1984).
- Giger, W., Molnar, E. & Schwarzenbach, R. P. *Rapports sur les Études et Recherches Entreprises dans le Bassin Lémanique*, 62-74 (Com. Int. Protection des Eaux du Léman, 1984).
- Pons, J. L., Rimbault, A., Darbord, J. C. & Leluan, G. *Annls Microbiol. (Inst. Pasteur)* **135B**, 219-222 (1984).
- Boyd, C. E. *Arch. Hydrobiol.* **72**, 1-9 (1973).
- Elsden, S. R., Hilton, M. G. & Waller, J. M. *Arch. Microbiol.* **107**, 283-288 (1976).
- Yokoyama, M. T. & Carlson, J. R. *Appl. Envir. Microbiol.* **41**, 71-76 (1981).
- Hunt, J. M., Miller, R. J. & Whelan, J. K. *Nature* **288**, 577-578 (1980).
- Button, D. K., Robertson, B. R. & Craig, K. S. *Appl. Envir. Microbiol.* **42**, 708-719 (1981).
- Williams, P. A. & Worsey, M. J. *J. Bact.* **125**, 818-828 (1976).

Kin selection and the problem of sperm utilization in social insects

Kenneth G. Ross

Department of Entomology, University of Georgia, Athens, Georgia 30602, USA

The evolution of social behaviour has posed a special problem for natural selection theory since Darwin. Sociality entails at least a partial loss of personal reproduction by some individuals, a seeming contradiction to the expectation that it be maximized by natural selection. The theory of kin selection^{1,2} seeks to explain social evolution by reference to high degrees of genetic relatedness among cooperating individuals. In the most diverse and conspicuous of social organisms, the social Hymenoptera (ants, bees, wasps), kin selection theory has been especially attractive because the male-haploid genetic system of the group leads to potentially high relatedness among female nestmates³⁻⁵. Multiple mating by queens poses a serious difficulty for kin selection, however, in that it reduces nestmate relatedness unless sperm from different males are used non-randomly⁶⁻¹⁰. This possibility has been investigated in only one highly social insect, the honey bee *Apis mellifera*, where the absence of suitable genetic markers has necessitated the use of artificial insemination^{10,11}. In this first study of long-term

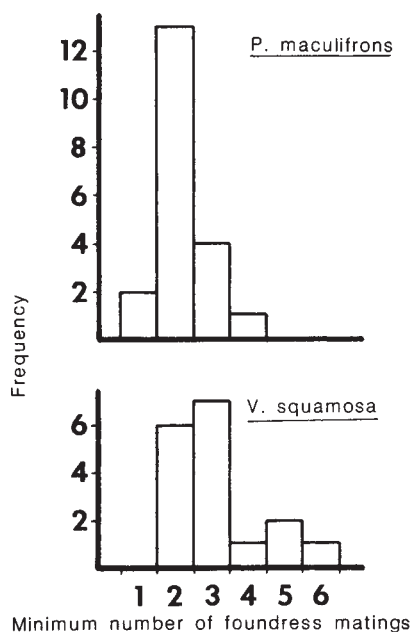


Fig. 1 Minimum number of matings by queens of two social wasp species, as determined from the diversity and distribution of multi-locus genotypes among female progeny. The ability to detect greater numbers of matings is constrained by the number of polymorphic genetic markers available for each species and the level of polymorphism (number and frequency of alleles) at each marker.

sperm utilization in natural populations of highly social insects I show that queens of two social wasp species use sperm from different males in relatively constant proportions through time, resulting in consistently low relatedness among female nestmates. I also show that workers do not supplement their inclusive fitness by reproducing directly in colonies in which the mother queen is present.

Colonies of the two species (20 colonies for *Paravespula maculifrons* and 17 colonies for *Vespula squamosa*) were collected in the southeastern U.S.A. during August–November of 1984 and 1985 and allozyme genotypes of colony members were determined electrophoretically. The mother queen was captured in 28 of the 37 colonies (76%); in the remainder her genotype was inferred from progeny genotypes. Genotype distributions indicate multiple insemination of at least 35 (94.6%) of the queens (90% of *P. maculifrons* and all *V. squamosa*). This is indicated by the presence of more than one or more than two genotypic classes among the female progeny of queens homozygous or heterozygous, respectively, at a marker locus. The effect of multiple insemination is to reduce relatedness among workers and, more importantly, relatedness between workers and the new queens they rear from the value of 0.75 that would be found in colonies headed by singly-mated queens to 0.320 for *P. maculifrons* and 0.403 for *V. squamosa* (Table 1).

The minimum number of matings per queen was determined from the distribution of multi-locus genotypes for her daughters; the modal minimum number is two matings for *P. maculifrons* and three for *V. squamosa* (Fig. 1). In conjunction with estimates for the harmonic mean numbers and insemination-effective numbers of matings derived from relatedness values (Table 1), it is evident that queens of the two species typically mate with 2–7 males before entering winter dormancy.

The brief period of multiple mating in the fall and the prolonged storage of sperm by queens for use the following year make it possible to study the effects of non-random usage of sperm from different males for fertilizations in a natural context. Such effects would be significant in that they increase relatedness among nestmates present at a given time and thus restore conditions favourable to the action of kin selection^{8,10}. Any male achieving a disproportionate representation of his sperm in

Table 1 Relatedness and number of matings for two species of social wasps

Species	No. colonies	$r(\pm s.e.)$	$\bar{H}$	m_1	m_2
<i>P. maculifrons</i>	20	0.320 ± 0.060	7.14	5.43	5.36
<i>V. squamosa</i>	17	0.403 ± 0.073	3.27	2.13	1.96

Average intra-colony relatedness among females (r), harmonic mean number of matings by queens ($\bar{H}$), insemination-effective number of matings (m_1) and short-term effective number of matings (m_2) are given. Relatedness was estimated from the distributions of genetic markers in colonies²³, and represents the mean for the three and two most polymorphic loci in *P. maculifrons* and *V. squamosa*, respectively. Harmonic mean numbers of matings were calculated from relatedness values^{10,24}, and assume equal and constant proportions of fertilizations by each male. These values indicate the maximum number of matings consistent with given relatedness values. These estimates for numbers of matings were adjusted downwards (effective numbers of matings) by taking into account variance in male contributions to fertilizations evident from point samples (m_1) and over time (m_2), and assuming that each unique sperm haplotype is contributed by a single male. Differences between m_1 and m_2 reflect the importance of intraspermatic events (for example, incomplete sperm mixing or differential viability) leading to changes in proportions of fertilizations by each male and concomitant changes in nestmate relatedness through time^{10,11}. Genetic markers were generated using starch gel electrophoresis²⁵. Six polymorphic loci were found for *P. maculifrons* (*Pgm-1,3*; *Hbdh*; *Mpi*; *Agp-1,2*) and four for *V. squamosa* (*Pgm-1*; *Est-1,2*; *Hbdh*). Mendelian inheritance of the enzyme products was confirmed by the predicted banding patterns among presumed progeny, banding patterns in heterozygotes consistent with known quaternary structures, and single bands for (haploid) males. A mean of 50.3 ± 13.7 (s.d.) workers, 82.0 ± 39.0 males and 37.9 ± 18.2 daughter queens were studied per colony. Genotype frequency distributions at polymorphic loci did not differ significantly between workers and queens in 27 of 28 (96.4%) comparisons (χ^2 test, all $p > 0.05$), consistent with a trophic mode of caste determination in vespines²⁶. Relatedness between workers and queens is thus essentially identical to relatedness among workers, and genotypes for the two castes were pooled for the relatedness estimates.

late-season fertilizations would secure a fitness advantage, as most eggs fertilized at this time are reared as queens.

Sperm utilization patterns in the wasps were studied by sampling foraging workers from eight colonies (five *V. squamosa*, three *P. maculifrons*) at regular intervals during the latter part of the season. Haplotypes of sperm used for fertilizations were inferred from worker and mother queen genotypes. Typical adult worker lifespans are shorter than the sample intervals¹², so the samples of workers did not overlap in age. Use of sperm from different males appears remarkably constant in several of the study colonies (Fig. 2), with few changes in the ordering of haplotype fertilization proportions between the beginning of sampling and season's end evident in any of the colonies. All but the rarest haplotypes are represented at every sample. Thus sperm from different ejaculates probably mix freely in the queens' spermathecae (sperm storage organs) and are used randomly for fertilizations. Variation in male contributions to fertilizations through time does not alter relatedness patterns in these social wasp colonies significantly, since the differences in estimates of effective numbers of matings (m_1 and m_2) for each of the species are small (Table 1). Thus workers present throughout the season have similar relatedness to and a common genetic interest in the new queens reared in autumn, and individual males cannot gain a fitness advantage by having their sperm used preferentially for the production of these queens.

Theoretical models^{13–15} indicate that under circumstances of low inclusive fitness effects among workers due to multiple mating of queens, conditions favourable to social evolution may be maintained by the retention of a measure of personal fitness among the workers, that is, by their directly producing some of the colony males (from unfertilized eggs). Although the ability

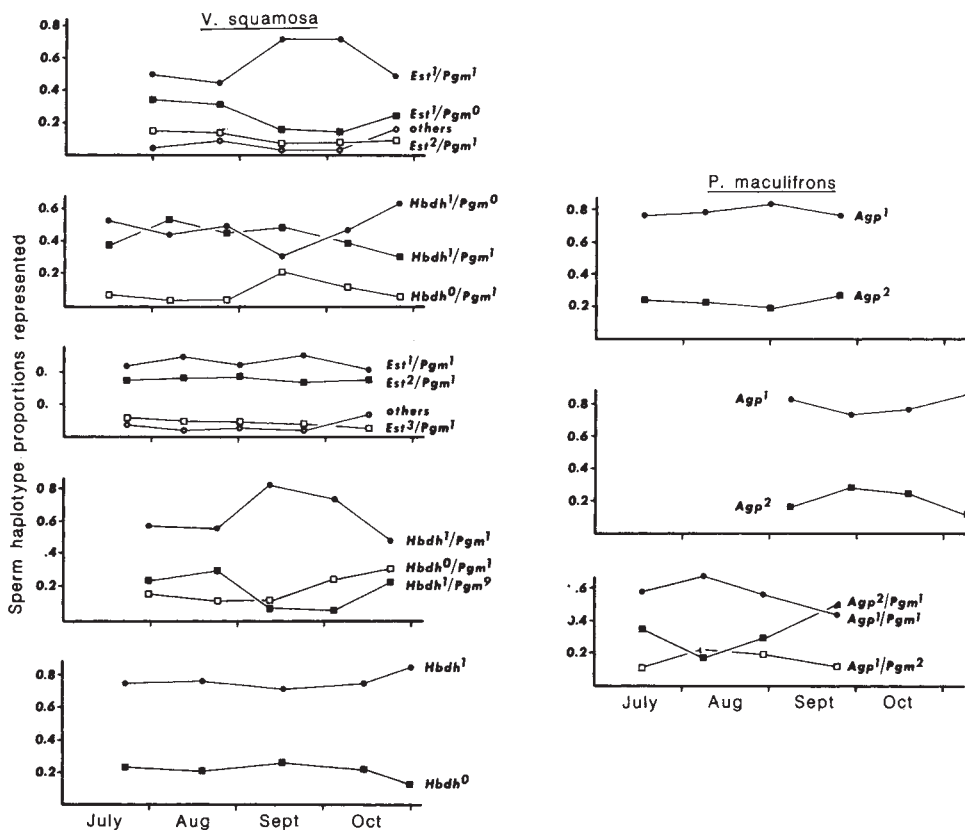


Fig. 2 Temporal sperm use patterns for queens of five colonies of *V. squamosa* and three colonies of *P. maculifrons*. Sperm haplotype proportions represented by each sample point were determined from worker genotype distributions (50 workers collected per sample) and genotypes of mother queens (collected at the final samples). Haplotypes are labelled with the diagnostic alleles as superscripts of informative marker loci. Numerical designations of alleles refer to the relative mobilities of the enzyme products in electrophoretic gels. New adult queens of the two species first appear in nests from late September to mid-October in the study area^{27,28}.

of workers to produce such males in the presence of the queen is poorly known for most social Hymenoptera¹⁶, several authors have speculated that many or all of the males in queenright vespine colonies may be worker offspring¹⁷. Twenty-three study colonies with males also contained workers and mother queens with genotypes appropriate for determining parentage of the males. In these colonies queens were homozygotes while all or a substantial proportion (>10%) of workers were heterozygous at one or more marker loci. Of 2,347 males studied from such queenright colonies, none were found to possess genotypes inconsistent with their being progeny of the queen. On the other hand, in three *V. squamosa* colonies that had evidently been queenless for some time (that is showing irregular brood patterns, extra eggs in cells and discoloration of worker gastral terga), substantial numbers of males were found with genotypes indicating that they were offspring of workers rather than the queen. Thus it appears unlikely that workers in these species reproduce directly when the queen is present in the nest.

I have shown here that workers in these wasp societies have relatively low inclusive fitness effects due to multiple mating by the mother queens, and also that the workers' personal fitness is essentially zero because they cannot reproduce directly in queenright colonies. Together with findings from other highly social groups^{10,18}, these results demonstrate that neither high levels of nestmate relatedness nor direct worker reproduction are necessary for the maintenance of advanced eusociality in Hymenoptera. Alternative explanations that emphasize constrained options of morphologically specialized workers; maternal manipulation, or factors extrinsic to the colony may help to explain the evolutionary stability of sociality in these advanced forms¹⁹⁻²¹. It is worth considering that sociality may evolve under conditions of single mating (with kin selection playing an important role), with a later switch to multiple mating occurring after worker differentiation (see refs. 7, 10, 22 for discussions of factors leading to multiple mating in social Hymenoptera). In this view the origin of sociality was mediated

by kin selection whereas its subsequent elaboration has been, to some extent, uncoupled from the necessity for close kinship, although still constrained by the specialized morphologies and behaviours of the participants. Close analogies may exist in other social organisms exhibiting diverse social structures.

I thank D. J. C. Fletcher, M. A. Moran, E. L. Vargo, M. S. Blum, R. W. Matthews and P. Pamilo for support and helpful criticisms, J. T. Brooks and S. U. Hall for technical help and B. May for electrophoresis expertise. Special thanks to the citizens of Athens for their cooperation.

Received 15 May; accepted 15 July, 1986.

1. Hamilton, W. D. J. *theor. Biol.* **7**, 1-52 (1964).
2. Maynard Smith, J. *Nature* **201**, 1145-1147 (1964).
3. Wilson, E. O. *The Insect Societies* (Belknap, Cambridge, 1971).
4. Crozier, R. H. in *Social Insects*, Vol. 1 (ed. Hermann, H.) 223-287 (Academic, New York, 1979).
5. Starr, C. K. in *Social Insects*, Vol. 1 (ed. Hermann, H.) 35-79 (Academic, New York, 1979).
6. Hamilton, W. D. A. *Rev. Ecol. Syst.* **3**, 193-232 (1972).
7. West-Eberhard, M. J. *Q. Rev. Biol.* **50**, 1-33 (1975).
8. Crozier, R. H. & Brückner, D. *Am. Nat.* **117**, 561-563 (1981).
9. Starr, C. K. in *Sperm Competition and the Evolution of Animal Mating Systems* (ed. Smith, R. L.) 427-464 (Academic, New York, 1984).
10. Page, R. E. A. *Rev. Entomol.* **31**, 297-320 (1986).
11. Laidlaw, H. H. & Page, R. E. *Genetics* **108**, 985-997 (1984).
12. Archer, M. E. in *Social Wasps, Their Biology and Control* (ed. Edwards, R.) 172-207 (Rentokil, East Grinstead, 1980).
13. Iwasa, Y. *J. theor. Biol.* **93**, 125-142 (1981).
14. Bartz, S. H. *Behav. Ecol. Sociobiol.* **11**, 223-228 (1982).
15. Pamilo, P. *Behav. Ecol. Sociobiol.* **15**, 241-248 (1984).
16. Fletcher, D. J. C. & Ross, K. G. A. *Rev. Entomol.* **30**, 319-343 (1985).
17. Ross, K. G. *J. Zool. (Lond.)* **205**, 411-424 (1985).
18. Ross, K. G. & Fletcher, D. J. C. *Behav. Ecol. Sociobiol.* **17**, 349-356 (1985).
19. West-Eberhard, M. J. in *Natural Selection and Social Behavior: Recent Research and New Theory* (eds Alexander, R. D. & Tinkle, D. W.) 3-17 (Chiron, New York, 1981).
20. Alexander, R. D. A. *Rev. Ecol. Syst.* **5**, 325-383 (1974).
21. Evans, H. E. *Bioscience* **27**, 613-617 (1977).
22. Crozier, R. H. & Page, R. E. *Behav. Ecol. Sociobiol.* **18**, 105-115 (1985).
23. Pamilo, P. *Genetics* **107**, 307-320 (1984).
24. Wade, M. J. *J. theor. Biol.* **95**, 351-368 (1982).
25. Ross, K. G., Fletcher, D. J. C. & May, B. *Biochem. Syst. Ecol.* **13**, 29-33 (1985).
26. Ishay, J. *Anim. Behav.* **23**, 425-431 (1975).
27. MacDonald, J. F. & Matthews, R. W. *J. Kans. ent. Soc.* **54**, 433-457 (1981).
28. MacDonald, J. F. & Matthews, R. W. *J. Kans. ent. Soc.* **57**, 134-151 (1984).

Trans-sexually grafted antennae alter pheromone-directed behaviour in a moth

Anne M. Schneiderman*, John G. Hildebrand†‡, Margaret M. Brennan§ & James H. Tumlinson§

* Department of Biology, Yale University, PO Box 6666, New Haven, Connecticut 06511, USA

† Arizona Research Laboratories Division of Neurobiology, 603 Gould-Simpson Science Building, University of Arizona, Tucson, Arizona 85721, USA

§ Insect Attractants, Behavior and Basic Biology Research Laboratory, Agricultural Research Service, US Department of Agriculture, Gainesville, Florida 32604, USA

‡ To whom correspondence should be addressed.

When tobacco hornworm moths (*Manduca sexta*) are tested in a wind tunnel with a source of female pheromones upwind, males but not normal females show pheromone-modulated anemotaxis and a characteristic mate-seeking behavioural sequence¹⁻⁷. These behaviours are produced by stimulation of sensory neurones found only in male antennae⁸⁻¹⁰. These neurones project axons only to dendrites of pheromone-specific interneurons^{11,12} in the macroglomerular complex, a region of neuropil in the antennal lobe characteristic of males but not present in normal females¹³⁻¹⁵. Some interneurons in the antennal lobes of female moths that have received grafts of male antennae (gynandromorphs) respond postsynaptically to stimulation with bombykal, a major component of the pheromone¹⁶. They branch into a region resembling the macroglomerular complex¹⁶, like their counterparts in normal males. We show here that gynandromorphic females respond to

pheromonal stimulation with anemotaxis. We also find that normal females display a similar sequence in response to the odour of their egg-laying site, the tobacco plant. It is likely that a common motor path is used either by pheromone-specific interneurons in the antennal lobes of males or by tobacco-specific interneurons in females. We assume that the interneurons in gynandromorphic females that branch into the macroglomerular complex induced by a grafted male antenna can activate this pathway.

An antennal imaginal disk was surgically extirpated or transplanted from a donor to a host fifth- (last) instar caterpillar¹⁶. A grafted disk forms an entire antenna, including sensory neurones which differentiate normally^{8,17} during pupal-adult metamorphosis. Their synaptic targets, the antennal-lobe neurones^{13,18}, arise from neuroblasts¹⁹ in the antennal lobes of the host. In moths with grafted antennae the glomerular neuropil of the antennal lobe is a mosaic of processes from host- and graft-derived cells. When the graft is from a donor of the opposite sex the glomeruli contain both male and female neural elements. A macroglomerular complex forms in the glomerular neuropil of both female and male antennal lobes innervated by antennal nerves from male grafts, and interneurons that arborize there receive synaptic input from pheromone receptors in the transplanted antenna¹⁶.

We tested 714 unoperated and 113 experimental moths (Table 1). After testing, we confirmed the sex of the moths, killed them, removed each antenna and noted whether it was morphologically 'male' or 'female', dissected the brains and prepared serial 8- μ m paraffin sections of the antennal lobes for light microscopy. Sections were stained by a modification of Bodian's Protargol method²⁰ or with haematoxylin and eosin. Only moths in which the antennal nerve from a graft either substantially innervated the ipsilateral antennal lobe or completely failed to contact the brain were included in the study. We eliminated moths with:

Table 1 Reproductive behaviour of moths (*M. sexta*) exposed to experimental odour sources in a wind tunnel

Group	Antennal perturbation	Odour source	Sex	Antenna 1	Antenna 2	MGC present AL 1	MGC present AL 2	n	RF (%)	TAX (%)	HOV (%)	T (%)	ES (%)	MA (%)	OB (%)	OVP (%)
1	Bilat Gyn	Pher	F	M	M	+	+	16	100	38	31	31	—	—	25	6
			M	F	F	—	—	7	100	43	—	—	—	—	—	—
2	Unilat Gyn	Pher	F	F	M	—	+	19	100	5	5	5	—	—	5	—
			M	F	M	—	+	4	100	75	50	50	—	—	—	—
3	Unilat deant	Pher	F	F	None	—	Uninn	5	100	—	—	—	—	—	—	—
			M	M	None	+	Uninn	23	100	87	87	78	48	—	—	—
4	Unilat uninn	Pher	F	F	M	—	Uninn	14	100	—	—	—	—	—	—	—
5	Gyn & deant	Pher	M	F	None	—	Uninn	5	100	40	—	—	—	—	—	—
6	Bilat deant	Pher	M	None	None	Uninn	Uninn	5	100	20	—	—	—	—	—	—
7	Bilat same-sex	Pher	F	F	F	—	—	5	100	20	—	—	—	—	—	—
8	Sham	Pher	M	M	M	+	+	3	100	100	100	67	67	—	—	—
			F	F	F	—	—	3	100	—	—	—	—	—	—	—
9	Control	Vrg-F	M	M	M	+	+	4	100	100	100	100	50	—	—	—
10	Control	Pher	M	M	M	+	+	38	100	87	82	82	82	82	—	—
			F	F	F	—	—	75	100	—	—	9*	—	—	—	—
11	Control	Tob	M	M	M	+	+	412	100	89	89	85	73	2	—	—
			F	F	F	—	—	40	100	90	90	85	—	—	85	63
12	Control	Hex	M	M	M	+	+	15	100	7	—	—	—	—	—	—
			M	M	M	+	+	134	100	2	—	—	—	—	—	—

Bilat, bilateral transplantation or extirpation of antennal disks; Deant, deantennated, disk extirpated; Gyn, gynandromorphic, donor and host of opposite sex; Same-Sex, donor and host of same sex; Sham, sham-operated, customary incisions made in larval head capsule, but disk left in place; Unilat, unilateral transplantation or extirpation of disk. Hex (hexane solvent); Pher (pheromone); Tob (tobacco); Vrg-F (virgin female). Responses; ES, displayed male erectable scales; HOV, hovered near source; MA, attempted to mate; OB, oviposition behaviour; OVP, laid egg on source; RF, random flight; T, touched source; TAX, upwind taxis toward source; *, random touch, preceded by random flight, rather than by taxis and hovering. AL, antennal lobe; MGC, macroglomerular complex; Uninn, uninnervated, antenna present but antennal nerve does not innervate AL; +, yes or behavioural response observed; —, no or behavioural response not observed; F, female; M, male; n, number of subjects. Behavioural testing was conducted in a 1 × 1 × 2.9 m Plexiglass wind tunnel²⁷⁻²⁹ with a canvas floor painted with alternating transverse black and white stripes. At the upwind end filtered air at 26 °C was blown through several layers of cheesecloth. Wind flowed through the tunnel at ~0.3 m s⁻¹ and was vented at the downwind end by an exhaust fan. We estimated the location and dimensions of the typical plume from an experimental odour source by releasing titanium tetrachloride smoke at the standard odour-release point (36 cm above the floor, 24 cm from the upwind end). The pheromonal mixture was obtained by iso-octane extraction of the terminal segments (containing pheromone glands) of the abdomens of virgin females 1-4 days after eclosion. It was diluted with hexane, applied at a concentration of 2 female-equivalents (the computed average quantity from the glands of two females) in 0.25 ml solvent to a 7-cm circle of construction paper folded into a triangle, and suspended at the upwind end of the wind tunnel. Pheromone mixtures from several extraction batches were used. Each batch was checked to confirm that 2 female-equivalents gave the same percentage (usually 100%) of positive responses from control male moths. To test responses of moths to naturally released pheromones, we placed calling virgin females on a wire-mesh perch at the upwind end of the tunnel. To test responses to tobacco, single large leaves (~18 × 30 cm) with stems in water were placed upright at the upwind end. All moths tested were unmated. They were separated by sex, housed in separate rooms in 16 h light: 8 h dark, and tested at light intensities of ~1.5 lx 1-5 h after the beginning of the dark phase. Controls were tested once 1-4 days after eclosion. Experimentals were usually tested on at least 2 successive days 1-4 days after eclosion, and the highest of the 2 or more scores was used. Moths that did not fly or had damaged wings or other obvious deformities were discarded.

antennae shorter than half the normal length; partially or fully regenerated host antennae; antennal nerves innervating ectopic sites in the brain; stunted antennal nerves or antennal lobes; or antennal lobes damaged by dissection.

The brains of seven normal females and three normal males, included in the group of experimental moths as 'blind' controls, had normal antennal nerves and antennal lobes. We assumed that the brains of the remaining 108 control (Table 1, groups 10, 11) females and 596 control (groups 9–12) males were normally innervated. Sections of brains from 11 males with one antenna removed (group 3) confirmed that the antennal lobe on the operated side was uninnervated while the control antennal lobe on the unoperated side was innervated normally by the antennal nerve from the intact antenna. We assumed this to be true for the remaining 12 males in group 3.

We judged the sexual dimorphism of the antennal lobes according to criteria described previously¹⁶. The anatomy of the host antennal lobes corresponded to the type of graft received without exception: macroglomerular complexes were present in antennal lobes innervated by male antennae but absent in those innervated by female antennae. Uninnervated antennal lobes had the stunted appearance characteristic of de-antennated antennal lobes²¹.

Odour sources used in the wind tunnel were live females, tobacco leaves or paper disks impregnated with either pure hexane or a hexane solution of the pheromone blend extracted from female pheromone glands (see Table 1 for methods). After placing a source in the tunnel, we introduced single moths at the downwind end and observed them for a maximum of 3 min (controls) or 5 min (experimentals).

Pheromone-modulated anemotaxis in *M. sexta* consists of random flight, anemotactic zig-zagging flight in the odour plume that narrows as the male approaches the pheromone source, diminished rate of forward advance or hovering within 10 cm of the source, touching the source, bending the abdomen and exposure of the erectable tufts of scales on the abdomen and attempted mating. A high percentage of normal males tested with either a calling female or a disk bearing the pheromone blend (Table 1, group 9 and males in group 10) completed the characteristic behavioural sequence up to 'touching', indicating that pheromone extracts are as effective as calling females in triggering mate-seeking behaviour. Male moths with both antennal lobes innervated by female grafts (males in group 1) or both antennal lobes uninnervated (group 6) did not perform any part of the sequence, in contrast to most of the males with at least one antennal lobe innervated by a male antenna (males in groups 2, 3, 7–10). Most (98%) of the males exposed to an odour plume from pure hexane (group 12) flew randomly, the other 2% showing only brief upwind excursions.

We have observed that normal females (group 11) flying in the plume from a tobacco leaf perform a sequence of behaviours very similar to that of males in a pheromonal plume: random flight, upwind zig-zagging in the plume, diminished rate of advance and hovering near or while touching the leaf. Females curve their abdomens and, on contact with the tobacco leaf, engage in oviposition behaviour or actually lay eggs. Only 7% of normal males (group 11) tested displayed taxis in response to tobacco and none approached the leaf.

Females that were unoperated (group 10), sham-operated (group 8) or had one or both antennal lobes uninnervated or innervated by antennae grafted from another female (groups 3, 4, 7) displayed none of the components of pheromone- or tobacco-modulated anemotaxis when tested with pheromones, other than very brief periods of upwind flight. Most of these females flew randomly, and those that showed upwind excursions only occasionally zig-zagged and soon reverted to random flight. None exhibited the sustained upwind progress typical of normal females in response to tobacco, or of males with at least one male antenna in response to pheromone. Seven control females (group 10) touched the pheromonal lure briefly follow-

ing random flight, but did not show the typical taxis or approach behaviours. By contrast, female gynandromorphs with one (group 2) or both (group 1) antennal lobes innervated by axons from grafted male antennae sometimes showed pheromone-modulated anemotaxis (5 out of 16 bilateral and 1 out of 19 unilateral gynandromorphs, Table 2). They flew upwind in the characteristic, zig-zagging pattern, hovered around and touched the pheromone lure, and made abdominal bending movements. One (Table 2, 33.485) laid eggs on the lure.

Table 2 Reproductive behaviour of gynandromorphic female moths exposed to female pheromones in a wind tunnel

Female	Antenna		MGC present		Age (days)	RF	TAX	HOV	T	OB	OVP
	1	2	AL 1	AL 2							
819	M	M	+	+	2	+	–	–	–	–	–
					3	+	+	+	+	+	–
839	M	M	+	+	1	+	–	–	–	–	–
					2	+	+	–	–	–	–
					3	+	+	+	+	+	–
883	M	F	+	–	1	+	–	–	–	–	–
					2	+	+	+	+	+	–
11.384	M	M	+	+	1	+	–	–	–	–	–
					3	+	+	+	+	–	–
					4	+	–	–	–	–	–
50.485	M	M	+	+	1	+	–	–	–	–	–
					2	+	+	+	+	+	–
33.485	M	M	+	+	1	+	–	–	–	–	–
					2	+	–	–	+	+	–
					3	+	+	+	+	+	+
					4	+	+	+	+	+	+

For abbreviations used, see Table 1.

Our findings show that the central processing of sensory information elicits anemotaxis and other reproductive responses to sex pheromones in female moths with grafted male antennae. In these females the male, pheromone-sensitive receptor cells in the transplanted antenna make connections that significantly influence the central nervous systems of the female hosts. They respond to sex pheromones with behaviours resembling both normal female tobacco-seeking and oviposition behaviours, and male mate-seeking and copulatory behaviours. Females with bilateral male antennal grafts are capable of normal oviposition behaviour as well as abnormal, pheromone-modulated flight behaviour. When housed with males in cages containing tobacco they mate and lay fertile eggs on the plants (I. Harrow, R. Kovelman, A.M.S. & J.G.H., unpublished observations). We do not know why only some of these females responded to pheromones. Further physiological testing of the antennal-lobe neurones may reveal subtle distinctions between gynandromorphs that do and those that do not respond to pheromones. By such testing and by careful observation of their anemotactic behaviours, we hope to discover any additional functions in reproduction that may be served by the sexually dimorphic antennal-lobe neurones and the protocerebral neurones with which at least some of them connect^{12–14,22–26}, and whether these functions are common to both sexes.

We thank Dr Patrick Greaney for use of laboratory facilities for some of these studies and Ruth Montague for technical assistance. This research was conducted while A.M.S. and J.G.H. were in the Department of Biological Sciences, Columbia University, and was supported in part by NSF grants (BNS-80-13511 and BNS-83-12769) and NIH grants (AI-16150 and AI-17711) to J.G.H. and by an NSF predoctoral fellowship and a PHS traineeship to A.M.S. A preliminary account of this work appeared in ref. 11.

Received 14 April; accepted 1 September 1986.

- Kennedy, J. S. & Marsh, D. *Science* **184**, 999–1001 (1974).
- Kennedy, J. S., Ludlow, A. R. & Sanders, C. J. *Nature* **288**, 475–477 (1980).
- Kennedy, J. S., Ludlow, A. R. & Sanders, C. J. *Physiol. Ent.* **6**, 395–412 (1981).
- Marsh, D., Kennedy, J. S. & Ludlow, A. R. *Physiol. Ent.* **6**, 225 (1981).
- Teal, P. E. A., McLaughlin, J. R. & Tumlinson, J. H. *Ann. ent. Soc. Am.* **74**, 324–330 (1981).
- Kuenen, L. P. S. & Baker, T. C. *Physiol. Ent.* **8**, 277–289 (1983).

7. David, C. T., Kennedy, J. S. & Ludlow, A. R. *Nature* **303**, 804-806 (1983).
8. Sanes, J. R. & Hildebrand, J. G. *Dev. Biol.* **51**, 282-299 (1976).
9. Kaissling, K.-E. in *Chemical Ecology: Odour Communication in Animals* (ed. Ritter, F. J.) 43-56 (Elsevier, Amsterdam, 1979).
10. Kaissling, K.-E. & Thorson, J. in *Receptors for Neurotransmitters, Hormones, and Pheromones in Insects* (eds Sattelle, D. B., Hall, L. M. & Hildebrand, J. G.) 261-282 (Elsevier, Amsterdam, 1980).
11. Schneiderman, A. M. thesis, Harvard Univ. (1984).
12. Christensen, T. A. & Hildebrand, J. G. *Soc. Neurosci. Abstr.* **10**, 862, (1984).
13. Matsumoto, S. G. & Hildebrand, J. G. *Proc. R. Soc. B213*, 249-277 (1981).
14. Boeckh, J., Boeckh, V. & Kühn, A. *Olfaction Taste, Paris* **6**, 315-321 (1977).
15. Olberg, R. M. *Physiol. Ent.* **8**, 419-428 (1983).
16. Schneiderman, A. M., Matsumoto, S. M. & Hildebrand, J. G. *Nature* **298**, 844-846 (1982).
17. Hildebrand, J. G. in *Model Neural Networks and Behavior* (ed. Selverston, A. I.) 129-148 (Plenum, New York, 1985).
18. Tolbert, L. P. & Hildebrand, J. G. *Proc. R. Soc. B213*, 279-301 (1981).
19. Nordlander, R. H. & Edwards, J. S. *Wilhelm Roux Arch. dev. Biol.* **162**, 197-217 (1969).
20. Gregory, G. E. in *Neuroanatomical Techniques: Insect Central Nervous System* (eds Strausfeld, N. J. & Miller, T. A.) 75-95 (Springer, New York, 1980).
21. Hildebrand, J. G., Hall, L. M. & Osmond, B. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 499-503 (1979).
22. Burrows, M., Boeckh, J. & Esslen, J. *J. comp. Physiol.* **145**, 447-457 (1982).
23. Ernst, K. D. & Boeckh, J. *Cell Tissue Res.* **229**, 1-22 (1983).
24. Olberg, R. M. *J. comp. Physiol.* **152**, 297-307 (1983).
25. Hildebrand, J. G. & Montague, R. A. in *Mechanisms in Insect Olfaction* (eds Payne, T., Birch, M. & Kennedy, C. E. J.) 278-285 (Oxford University Press, London, 1986).
26. Light, D. M. in *Mechanisms in Insect Olfaction* (eds Payne, T., Birch, M. & Kennedy, C. E. J.) 287-301 (Oxford University Press, London, 1986).
27. Marsh, D., Kennedy, J. S. & Ludlow, A. R. *Physiol. Ent.* **3**, 221-240 (1978).
28. Miller, J. R. & Roelofs, W. L. *J. chem. Ecol.* **4**, 187-198 (1978).
29. Baker, T. C. & Linn, C. E. Jr in *Techniques in Pheromone Research* (eds Hummel, H. E. & Miller, T. A.) 75-110 (Springer, New York, 1984).

Anatomical, cellular and molecular analysis of 8,000-yr-old human brain tissue from the Windover archaeological site

Glen H. Doran*, David N. Dickel*, William E. Ballinger Jr†, O. Frank Agee‡, Philip J. Laipis§¶ & William W. Hauswirth||

* Department of Anthropology, Florida State University, Tallahassee, Florida 32306, USA

Departments of † Pathology, ‡ Radiology, § Biochemistry and Molecular Biology, and || Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida 32610, USA

Recovery and analysis of ancient tissue and bone of human origin has long been extensively investigated. Only recently, however, has it been technically possible to recover genetic material from ancient human¹ and animal² samples. As both previous studies involved dried tissue, it is important to determine whether other conditions may also preserve ancient tissue and genetic material. We describe here an analysis of preserved human bone and soft matter discovered in 1984-85 buried in a small swampy pond in central Florida. The recovered skeletal material represented a minimum of 40 individuals of both sexes and various ages. Corrected radiocarbon dates directly from bone and from peat matrix gave consistent ages in the range of 7,790 to 8,290 yr before present (BP). Nine individuals with intracranial soft matter were recovered and, in five of these, material recognizable as preserved or replaced brain tissue was present. Further analysis demonstrated gross anatomical features, remnant cellular structure and human DNA. As this find appears to be the oldest-known example of preserved human cell structure and DNA, it represents a significant resource for both anthropological and genetic studies.

The Windover pond (8BR246) is in Brevard County, Florida, USA. The pond (60 × 80 m) seasonally fluctuates in depth to a maximum of 1 m and contains a soft bottom composed of several distinct peat strata, total depth 4-5 m (Fig. 1). The uppermost black, sawgrass peat (W. Spackman and R. G. Holloway, personal communications) has been dated at 4,790 ± 100 BP (Beta

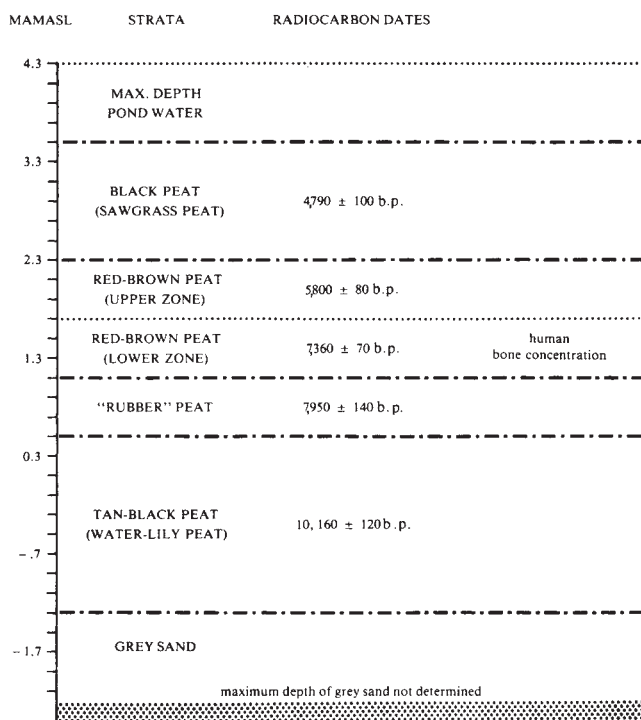


Fig. 1 Stratigraphic profile, peat types and uncorrected radiocarbon dates on peat from the Windover site, 8BR246. Radiocarbon dating was performed either by Isotracer Laboratory, University of Toronto, Canada, or by Beta Analytical, Coral Gables, Florida. Sample numbers are indicated in the text, for example, Beta 10763. Dates are in years BP. A correction factor of +800 yr should be added to dates between 7,000 and 10,000 yr BP¹⁰. MAMASL = metres above mean annual sea level. Vertical scale units, 20 cm per division.

10763) and is underlain by a red-brown peat stratum containing large amounts of well preserved flora and freshwater fauna. The upper zone of this stratum has been radiocarbon dated at 5,800 ± 80 yr BP (Beta 10764). The lower 50-cm zone of the red-brown peat contains most of the human skeletal material. Underlying the red-brown peat is a stratum of gelatinous 'rubber' peat dated at 7,950 ± 140 yr BP (Beta 10855). Under this is a tan-black, water lily peat dated at 10,160 ± 120 yr BP (Beta 11382), and below the peat is a grey Pleistocene sand.

Based on crania (adults) or crania and mandibles (subadults), at least 40 individuals were recovered. The recovered bone is strikingly well preserved and nonfriable. Nine recovered crania contained soft tissue remnants: four adults and one subadult with recognizable brains, and four other individuals (including three subadults of roughly 5-19 years of age) with amorphous masses of brain tissue mixed with peat. Bone fragments from one of the crania containing a brain were dated at 6,990 ± 70 yr BP (Isotracer TO-207) using an accelerator-mass spectrometry method on isolated collagen (1.5% by weight). Red-brown peat from inside a second cranium containing a recognizable brain has been dated at 7,410 ± 80 yr BP (Beta 11838), and peat, in direct contact with this cranium and associated bones, has been dated at 7,360 ± 70 yr BP (Beta 11381).

Based on the cranial structure, dental attrition and limited post-cranial skeletal evidence, the four adult crania were from a female at least 45 yr old and three males about 25-35 yr old. When the first female intracranial mass was removed, the immediate visual impression was of a human brain. However, it was extremely fragile and handling was difficult. The next crania to be recovered were appraised using conventional X-ray imaging, computerized axial tomography and proton magnetic resonance imaging (Fig. 2a). Chemically different components within the intracranial mass could be recognized and differenti-

¶ To whom correspondence should be addressed.

Fig. 2 A magnetic resonance image and anatomical sections of two intact male brains. *a*, A 1-cm thick magnetic resonance image of the medial portion of the adult male brain 57-300, sagittal section, with the specimen facing right. The skull has been removed and the brain is embedded in agar (uniform medium grey). The brain material is light grey. Several gross anatomical features are identifiable: 1, occipital pole; 2, frontal pole; 3, lateral ventricle; 4, cingulate gyrus. Images were produced by a Technicare Teslacon 0.15T resistive magnet unit. The machine and pulsing techniques are used in routine clinical practice. *b*, A coronal section through the agarose gel-embedded ancient brain of male 57-55, and a similar section of a contemporary brain. Although the old brain section is surrounded by peat and has undergone some fragmentation, many gross anatomical structures are present. The two cerebral hemispheres and their gyral patterns are clearly visible. 1, Interhemispheric fissure; 2, corpus callosum; 3, lateral ventricle; 4, insular cortex; 5, putamen; 6, internal capsule; 7, thalamus; 8, third ventricle. A subtle distinction between grey and white matter is still apparent.

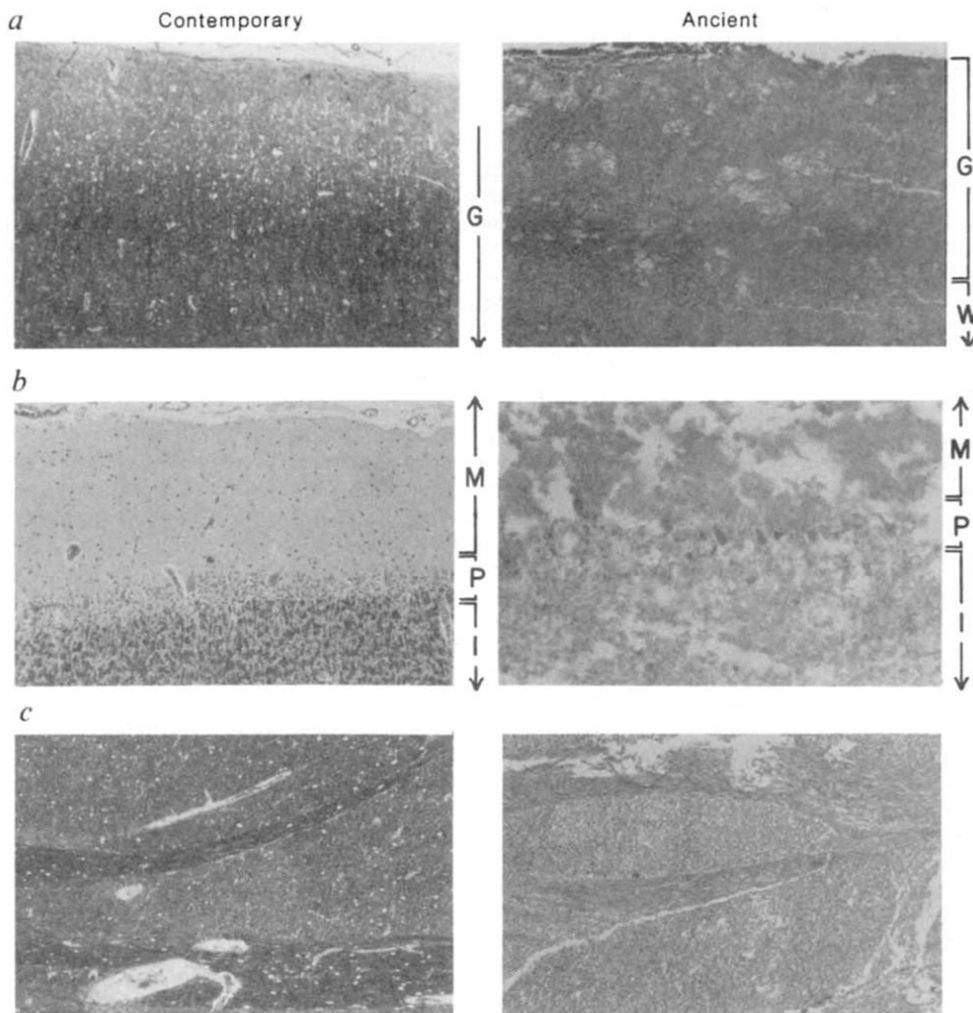
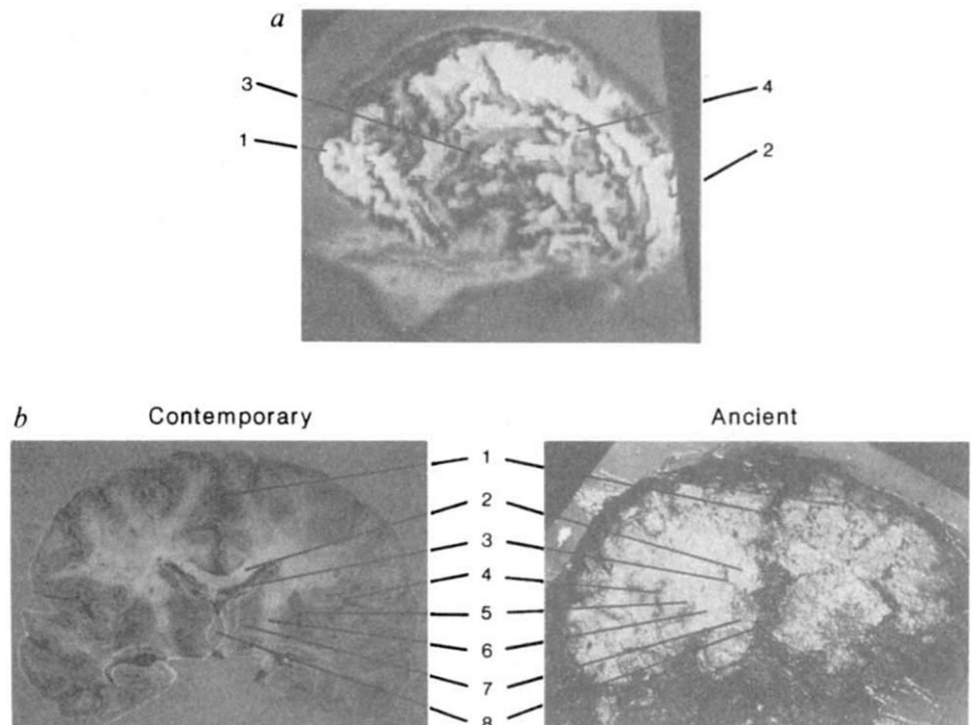
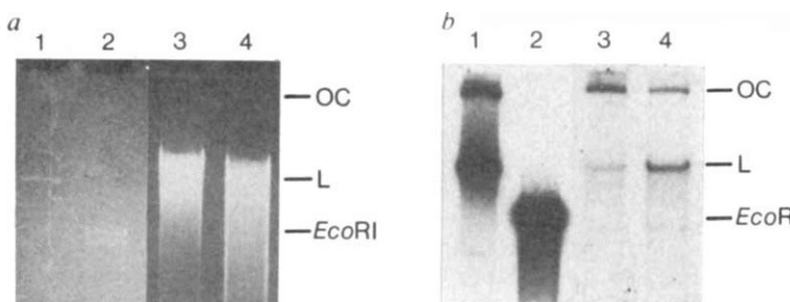


Fig. 3 Histological comparison of a contemporary brain with the ancient female brain. *a*, Sections of cerebral cortex visualized with silver axon stain¹¹. The surface of each brain is at the top. A small amount of peat is visible on the surface of the ancient brain. The division between grey and white matter is indicated on the ancient brain section. At the same magnification, the section of modern brain shows only grey matter, due to general tissue shrinkage suffered by the ancient brain. Vertical axonal fibres are visible in the grey matter of both sections. The small darkly staining particles in the ancient brain probably represent remains of neurones. (Seiver-Munger stain, $\times 100$). *b*, Sections of the cerebellum stained with haematoxylin and eosin¹¹. A correlation of contemporary and ancient molecular layers (M), Purkinje cell layers (P) and inner granular layers (I) are indicated on the right. Clear areas in the centre of most Purkinje cells in the ancient cerebellum are probably residual nuclei (contemporary section, $\times 250$; ancient section, $\times 400$). *c*, Section of the pons stained with Luxol fast blue for myelin¹¹. Cross-sectional views of cortical spinal tracts and longitudinal sections of ponto-cerebellar tracts are clearly evident in both (contemporary section, $\times 100$; ancient section, $\times 200$).

Fig. 4 Identification of human mtDNA sequences in DNA from an 8,000-yr-old adult male brain. Total DNA was isolated and purified as described below. An aliquot was digested with *Eco*RI and electrophoresed on a 1% agarose gel along with an undigested aliquot and samples of authentic human (KB cell) mtDNA treated similarly. After staining and photography, the gel was blotted and hybridized to a human mtDNA-specific probe⁵, washed and autoradiographed. Central lanes containing the enzymes, buffers and carrier tRNA used in the extraction and enzyme digestion showed no hybridization to the probe. *a*, Ethidium-stained gel. Lane 1, undigested KB cell mtDNA; lane 2, *Eco*RI-digested KB cell mtDNA; lane 3, undigested ancient DNA; lane 4, *Eco*RI-digested ancient DNA. OC, L, and *Eco*RI refer to the positions of the open (nicked) circular, linear and 8,050-base pair (bp) *Eco*RI (D-loop-containing) fragment of KB cell mtDNA, respectively^{6,7}. *b*, Autoradiograph of the blotted gel. Lanes 1 and 2 were exposed for 3 days, lanes 3 and 4 for 7 days. Lane identification as in *a*.



Methods. Ancient cerebral cortex (15 g) was homogenized (0.1 M EDTA, 0.15 M NaCl, 0.015 M Na-citrate pH 7.6, 1% SDS, and 4 mg yeast transfer RNA), and extracted three times with chloroform:octanol (5:1). The aqueous phase was extracted with ether and precipitated with 2.5 vols of -20° ethanol. The precipitate was resuspended, pronase digested, phenol extracted twice and reprecipitated. Nucleic acids were resuspended in caesium chloride-ethidium bromide and centrifuged to equilibrium in a SW 50.1 rotor (60 h at 35,000 r.p.m.). The visible ethidium-stained band was collected and ethanol precipitated. An aqueous solution of the precipitate was dark brown and was passed over two 10-ml Sephadex G-10 columns to remove pigmented material. The eluate was again ethanol precipitated and used for gene detection. Aliquots of ancient DNA (2 μ g) were digested with 20 U *Eco*RI for 10 h at 37° C. The digests were electrophoresed on 1% agarose gels along with a human (KB cell) mtDNA sample which was similarly treated. After electrophoresis, the gel was blotted to Biodyne transfer membrane (ICN) using the conditions suggested by the supplier. The blot was prehybridized in 50% formamide containing 0.45 M NaCl, 0.045 M Na-citrate pH 7.6, 1% SDS, 0.02% each Ficoll, polyvinylpyrrolidone, bovine serum albumin, 1 mM sodium pyrophosphate and 100 μ g ml⁻¹ torulla tRNA at 42° C for 16 h. An [α -³²P]GTP-labelled hybridization probe made from plasmid pK406-SP was used to identify human mtDNA specifically. This plasmid contains a 406-bp fragment unique to the human mtDNA D-loop^{5,12,13}, the most variable region of vertebrate mtDNA^{14,15}. This human mtDNA fragment does not hybridize to other mammalian mtDNA under our hybridization conditions. The cloned fragment was digested with *Pst*I, which cuts distal to the SP6 promoter. The 3' single-stranded end was removed with the Klenow fragment of *Escherichia coli* DNA polymerase I and the DNA transcribed using SP6 RNA polymerase (Promega Biotech). The RNA probe (1 $\times$ 10⁸ c.p.m. μ g⁻¹) was DNase treated and 5–10 $\times$ 10⁶ c.p.m. added to the prehybridized blot. Hybridization was carried out in prehybridization solution plus 4.8% dextran sulphate at 42° C for 16 h. The blot was washed twice for 5 min each at room temperature in 2 $\times$ SSC, 0.1% SDS, then twice for 45 min each in 0.2 $\times$ SSC, 0.1% SDS at 65° C. The washed blot was exposed for 3 and 7 days to XAR-5 film with a Cronex Lighting Plus intensifying screen.

ated using an arbitrary false-colour magnetic resonance image to enhance contrast. Living tissue gives a high signal intensity from soft tissue and a lower signal from dense connective tissue. When these brains were sectioned (Fig. 2), peat and brain tissue were clearly distinguishable.

Gross examination of the brain-like masses after removal from the skull disclosed the external gyral pattern of an atrophic human brain which had shrunk to about one-quarter of the original size. No meningeal coverings or blood vessels remained. The brain was tan-grey and had a soft granular consistency. The two cerebral hemispheres divided by the longitudinal fissure were identifiable, and the Sylvian fissure could be seen on either one or both hemispheres. Cerebellar tissue containing visible folia was present below the occipital lobes. However, the region of the brain stem in all cases was amorphous and finer structure could not be identified. Transverse slices of the remaining material exposed parietal, temporal and occipital lobes with peat filling all fissures. Internal structures such as the thalamus, basal ganglia and ventricular system were clearly visible (Fig. 2b). The overall impression from physical analysis of these intracranial masses was that, although shrunken and altered in consistency, the gross anatomical features of contemporary brains were present.

Representative samples were taken from the cerebral hemisphere, cerebellum and brain stem and processed for light and electron microscopy. The stained sections (Fig. 3a) resembled cerebral cortex and contained yellow granular pigment, often in pyramidal shapes, corresponding to the remains of neurones. The cyto-architectural pattern was similar to that of contemporary cerebral cortex tissue. Occasional processes consisting of parallel arrayed fibres extending to the cortical surface, as well as horizontal arrayed fibres within the subcortical white matter were present. Sections of cerebellum contained preserved Purkinje cells (Fig. 3b), arranged in the spatial pattern seen in contemporary cerebellar tissue. A section of the pons contained a typical pattern of crossing ponto-cerebellar tracts as well as

cortical spinal tracts divided by remains of pontine nuclei (Fig. 3c). Portions of each tissue were examined by transmission and scanning electron microscopy. The most striking finding was an accumulation of electron-dense bodies which corresponded to the yellow granular pigment seen by light microscopy (Fig. 3a). These granules may be residual lipofuscin pigment^{3,4}, consistent with our observation that there was significantly more pigment in the 45-yr-old female brain than in the 25-yr-old male brain. Histological analysis thus confirms the radiological and gross anatomical studies described above and shows that limited but definite cellular structure remains.

Nucleic acids were extracted from 15 g of relatively peat-free cortex which was solubilized and extracted with chloroform-phenol, then further purified by centrifugation in a CsCl-ethidium bromide density gradient. Material banding at a density of 1.55 g cm⁻³ was collected and identified as DNA by DNase sensitivity and RNase resistance. Figure 4a shows an ethidium bromide stained gel of this DNA. DNA of 8–20 kilobases (kb) was clearly present. To determine whether this DNA was of human origin, the gel was blotted and hybridized to a probe specific for human mitochondrial DNA⁵. The probe hybridized to appropriately sized species in both digested and undigested brain DNA, demonstrating that human mtDNA was present (Fig. 4b).

The total yield of DNA was only about 1 μ g per g tissue, 1% of that normally isolated from fresh tissue. In view of the significant loss of cell structure seen histologically, it is not surprising that only a small fraction of the DNA remains. A comparison of hybridization intensities of old DNA and human mtDNA with the mtDNA probe suggests that $\sim$ 0.05% of the total old DNA was mtDNA. DNA isolated from brain tissue would normally yield 0.5–1% mtDNA. This decrease could be due to either preferential mtDNA damage or loss during the extraction procedure. The DNA sample also contains significant amounts of nucleic acids derived from the surrounding plant material, as the contaminating peat contains about the same

amount of DNA (per weight) as brain tissue. However, plant DNA hybridizes to neither the human mtDNA probe nor a human *Alu* sequence probe.

The mtDNA digested poorly with restriction enzymes, as shown by the *EcoRI* digest (Figure 4b, lane 1), which does not exhibit the expected hybridizing 8-kb mtDNA fragment⁶. However, a significant conversion of open circular to linear molecules did take place, as expected if only a fraction of the *EcoRI* recognition sequences remained. Resistance to enzyme digestion is an intrinsic property of the old DNA as a bacterial plasmid DNA, mixed with an old DNA sample, was digested to completion. Similarly, old nuclear DNA was not easily digested, as similar blots probed with radiolabelled DNA and RNA representing nuclear sequences known to be present in multiple copies in the human genome, including an *Alu* sequence⁷ and the large and small ribosomal RNAs⁸ gave diffuse hybridization patterns, not distinct bands. This inability to digest the DNA completely is likely to be due to base modifications or other DNA damage leading to a loss of restriction enzyme site recognition. Preliminary results suggest that alkali-sensitive cleavage sites are present every few hundred base pairs (bp) in the old DNA, and we believe DNA damage is probably affecting both DNA digestion and hybridization efficiencies. DNA damage may also explain the presence of open circular mtDNA since inter-strand crosslinks, often formed after depurination in solution⁹, will stabilize circular forms even in the presence of multiple single-strand breaks.

An independent estimate of the fraction of human DNA sequences in the old DNA samples is given by the quantification of *Alu* sequences in dot blots (data not shown). We estimate that *Alu* sequences are present at 1% of the level seen in an equivalent amount of human placental DNA, again suggesting either co-isolation of plant DNA with human sequences or sufficient DNA damage to reduce hybridization efficiency. At present, we cannot exclude either possibility.

This brain tissue dates from the Early Archaic period¹⁶ (~8,000 yr ago) and is thus the oldest human soft tissue yet analysed at a molecular level. We have demonstrated the presence of human DNA and remnant cellular and anatomical structure in the tissue. It is especially noteworthy that this material has been preserved in an aqueous environment, suggesting that intact DNA can survive in other than extremely arid conditions, which greatly widens the sites where ancient genetic material may be found. We are now attempting to clone identifiable human genes or gene fragments.

We thank Windover Farms, Inc. and Jim Swann for their initial observation of the site, and for cooperation and financial support. This research was also supported by funds from the State of Florida. We thank Drs H. Baer, W. Bui, S. Khan, R. Roberts, K. Scott, P. Small and S. Tanhauser, also L. Ballinger, K. Dodge, K. Dukes, C. Fletcher, W. Gage, P. Mitchell, L. Peters and T. Stone for helpful comments and technical assistance.

Received 20 March; accepted 19 August 1986.

1. Pääbo, S. *Nature* **314**, 644-645 (1985).
2. Higuchi, R., Bowman, B., Friedberger, M., Ryder, O. A. & Wilson, A. C. *Nature* **312**, 282-284 (1984).
3. Taubold, R. D. *et al. Lipids* **10**, 383-390 (1975).
4. Adams, R. D. & Lee, J. C. in *Histology and Histopathology of the Nervous System* (eds Haymaker, W. & Adams, R. D.) 234-237 (Thomas, Springfield, 1982).
5. Chang, D. D. & Clayton, D. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 351-355 (1985).
6. Anderson, S. *et al. Nature* **296**, 457-465 (1981).
7. Houck, C. M., Rinehart, F. P. & Schmid, C. W. *J. molec. Biol.* **132**, 289-306 (1979).
8. Long, E. O. & Dawid, I. B. *Rev. Biochem.* **49**, 727-764 (1980).
9. Goffin, C., Bricteux-Grégoire, S. & Verly, W. G. *Biochim. biophys. Acta* **783**, 1-5 (1984).
10. Klein, H., Lerman, J., Damon, P. & Ralph, E. *Radiocarbon* **24**, 103-150 (1982).
11. Luna, L. G. *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology* 3rd edn (McGraw-Hill, New York, 1968).
12. Anderson, S. *et al. in Mitochondrial Genes* (eds Slonimsky, P., Borst, P. & Attardi, G.) 5-43 (Cold Spring Harbor Laboratory, New York, 1982).
13. Hixson, J. A. thesis, Univ. Michigan (1983).
14. Upholt, W. B. & Dawid, I. B. *Cell* **11**, 571-583 (1977).
15. Brown, W. W., George, M. Jr & Wilson, A. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1967-1971 (1979).
16. Mitani, J. T. & Charles, C. H. *Fairbank Florida Archaeology* **23** (Academic, New York, 1980).

Mapping human visual cortex with positron emission tomography

Peter T. Fox*†, Mark A. Mintun*,
Marcus E. Raichle*‡, Francis M. Miezin§,
John M. Allman§ & David C. Van Essen§

* Department of Radiology, † Department of Neurology and Neurological Surgery (Neurology) and ‡ McDonnell Center for Studies of Higher Brain Function, Washington University School of Medicine, St Louis, Missouri 63110, USA
§ Division of Biology, California Institute of Technology, Pasadena, California 91125, USA

Positron-emission tomography (PET) can localize functions of the human brain by imaging regional cerebral blood flow (CBF) during voluntary behaviour. Functional brain mapping with PET, however, has been hindered by PET's poor spatial resolution (typically >1 cm). We have developed an image-analysis strategy that can map functional zones not resolved by conventional PET images. Brain areas selectively activated by a behavioural task can be isolated by subtracting a paired control-state image from the task-state image, thereby removing areas not recruited by the task. When imaged in isolation the centre of an activated area can be located very precisely. This allows subtle shifts in response locale due to changes in task to be detected readily despite poor spatial resolution. As an initial application of this strategy we mapped the retinal projection topography of human primary visual cortex. Functional zones separated by less than 3 mm (centre-to-centre) were differentiated using PET CBF images with a spatial resolution of 18 mm. This technique is not limited to a particular brain area or type of behaviour but does require that the increase in CBF produced by the task be both intense and focal.

PET records the spatial distribution of an administered radio-nuclide in selected planes of the *in vivo* brain. Spatial resolution in PET images, however, is inherently limited. This limitation is described most simply by reference to point sources of radio-activity, as follows. Emission detection and image reconstruction blur a point source into an activity distribution or point-spread function¹ (Fig. 1). As two simultaneously imaged point sources cannot be discriminated when they lie closer together than the width of one point-spread function at its half-maximum amplitude (the full-width at half-maximum or FWHM), this is the usual measure of spatial resolution for PET (Fig. 1). The spatial resolution of most PET images is greater than 1.0 cm (FWHM).

A distinction can be drawn, however, between spatial resolution and source localization. Two adjacent point sources cannot be resolved when separated by less than one FWHM. A single point source, however, can be localized with a precision well below the FWHM (Fig. 1). This phenomenon is well known in signal detection theory² and has been postulated to underlie the property of hyperacuity in the human visual system³. In application to PET, this suggests that zones of brain activity separated by less than one FWHM could be distinguished from one another by sequential activation even though they would not be seen as distinct (not resolved) if activated simultaneously (Fig. 1).

Local alterations in neuronal electrical activity occur during task performance (such as stimulation) and induce local changes in CBF⁴. As CBF in nonactivated brain areas is quite stable over time⁵, a resting-state CBF image can be directly subtracted from a stimulated-state image to create a new image showing only the focal changes in CBF caused by the activation task. In subtraction format, a local CBF change appears as an isolated focus of high activity within a low-level background, quite similar to the image of an isolated point source. This provides a means for applying the principle described above. Brain areas lying closer together than one FWHM should be discriminable by activating each area in isolation and determining the location

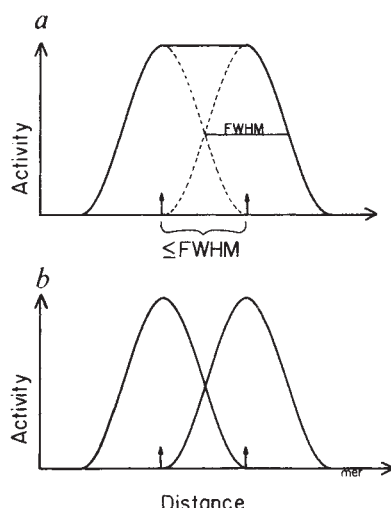


Fig. 1 Due to the limited spatial resolution of emission imaging, a point source of radioactivity is imaged as an activity distribution (point-spread function) composed of a central maximum surrounded by a radially declining skirt of activity. Spatial resolution traditionally has been defined by the width of the point-spread function at half-peak activity (full-width at half-maximum or FWHM). *a*, Two simultaneously imaged point sources cannot be resolved when separated by a distance of one FWHM or less. *b*, Isolated point sources localized with a precision well below the FWHM, allow sequentially imaged sources with a centre-to-centre separation much less than the FWHM to be distinguished by a difference in the location of the centres of the activity distributions.

of each response within subtraction-format images. As an initial application of this experimental strategy, we mapped the representation of the central retina in human primary visual cortex.

Test stimuli were checkerboard rings about a central fixation point (Fig. 2). Three eccentricities were tested: macular (0.1–1.5°), perimacular (1.5–5.5°) and peripheral (5.5–15.5°). The control stimulus was a fixation point only. Stimulus presentation began 30–40 s before scan initiation and continued throughout

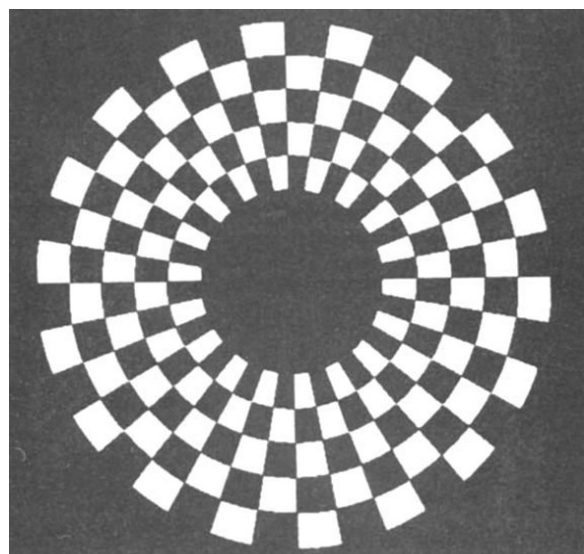


Fig. 2 All visual stimuli were checkerboard annuli with a central fixation point. The peripheral stimulus is shown and extended from 5.5 to 15.5°. The perimacular annulus extended from 1.5 to 5.5°. The macular annulus extended from 0.1 to 1.5°. Check size increased with eccentricity from 0.5 to 2.0°. Checks were red on black and alternated colours at 10 Hz to maximize the induced CBF response⁵.

scan acquisition. Seventeen test-control pairs of CBF scans were obtained from six normal volunteers: five macular, six perimacular, six peripheral.

Water labelled with oxygen-15 ($H_2^{15}O$), a positron-emitting radiotracer with a half-life of 123 s, was used to measure brain blood flow. This tracer was given as a bolus injection into a peripheral vein and circulated into the brain within 10–20 s. A 40-s scan recorded the distribution of the administered radioactivity within the brain, from which images of regional CBF (Fig. 3*a*) were calculated^{6,7}. A 10-min interscan interval was sufficient for isotope decay (five half-lives) and to reestablish resting levels of regional CBF within activated areas⁵. Images of regional CBF change (Fig. 3*b,c,d*) were formed by paired subtraction of control-state from stimulated-state CBF measurements⁸. Response foci were located by computer algorithm and plotted in stereotactic coordinates (Table 1)⁹.

Response locale varied systematically with stimulus field (Table 1). Macular responses were posterior and inferior, lying at the occipital pole (Fig. 3*b*). Peripheral responses were anterior and superior, lying on the medial occipital surface (Fig. 3*d*). Perimacular responses lay between macular and peripheral (Fig. 3*c*). Shifts in cortical response locale as small as 3 mm were readily detected and statistically significant (Table 1).

The cortical magnification factor, defined as the linear extent of striate cortex to which each degree of the retinal field projects^{10–12}, was calculated for three retinal eccentricities (Table 2) from the observed response locations (Table 1). As cortical

Table 1 Response locale in stereotactic coordinates

Stimulus field	Macula	Perimacula	Periphery
Degrees	0.1-1.5	1.5-5.5	5.5-15.5
<i>n</i>	5	6	6
Antero-posterior axis (cm)			
Mean (s.d.)	-6.86 (0.13)	-6.29 (0.09)	-5.96 (0.07)
Statistical analysis	ANOVA: $F = 42$, $P < 0.0001$ Newman-Keuls: Macula < perimacula < periphery, $P < 0.01$ for all inequalities		
Vertical axis (cm)			
Mean (s.d.)	-0.50 (0.36)	-0.12 (0.32)	-0.64 (0.42)
Statistical analysis	ANOVA: $F = 90$, $P < 0.00001$ Newman-Keuls: Macula < perimacula < periphery $P < 0.01$ for all inequalities		

Degrees indicates the inner and outer boundaries of an annular stimulus in degrees of eccentricity from the fixation point. Response foci were located by a three-dimensional search for the centre of the maximum response (peak CBF change) using a roving cubic (7.0 cm³) region of interest and a centre-of-mass algorithm. All responses lay in the midsagittal plane. As significant shifts in response locale occurred only along the antero-posterior and vertical axes, only these dimensions are reported. Distances are in cm from the centre of the AC–PC line in proportionately measured stereotactic coordinates⁹.

infolding and curvature of the calcarine fissure were not visualized, these values set a lower boundary rather than a mean.

Our observations confirm and extend prior studies of human visual cortex. Case reports of patients with occipital lobe lesions established the general outline of retinal:cortical projection topography, but lacked accurate descriptions of lesion locale and were limited by the stereotypy of spontaneous lesions^{13–16}. Plots of the perceived location of visual illusions (phosphenes) induced by electrical stimulations of the occipital cortex during neurological surgery have been reported, but gave irregular and discontinuous maps^{17,18}. Evoked magnetic field recordings have detected field-source shifts in response to shifts in stimulus eccentricity, but could not specify unequivocally the anatomical sites of the fields' sources and needed rather gross stimuli to evoke measurable fields¹⁹. The PET imaging strategy presented

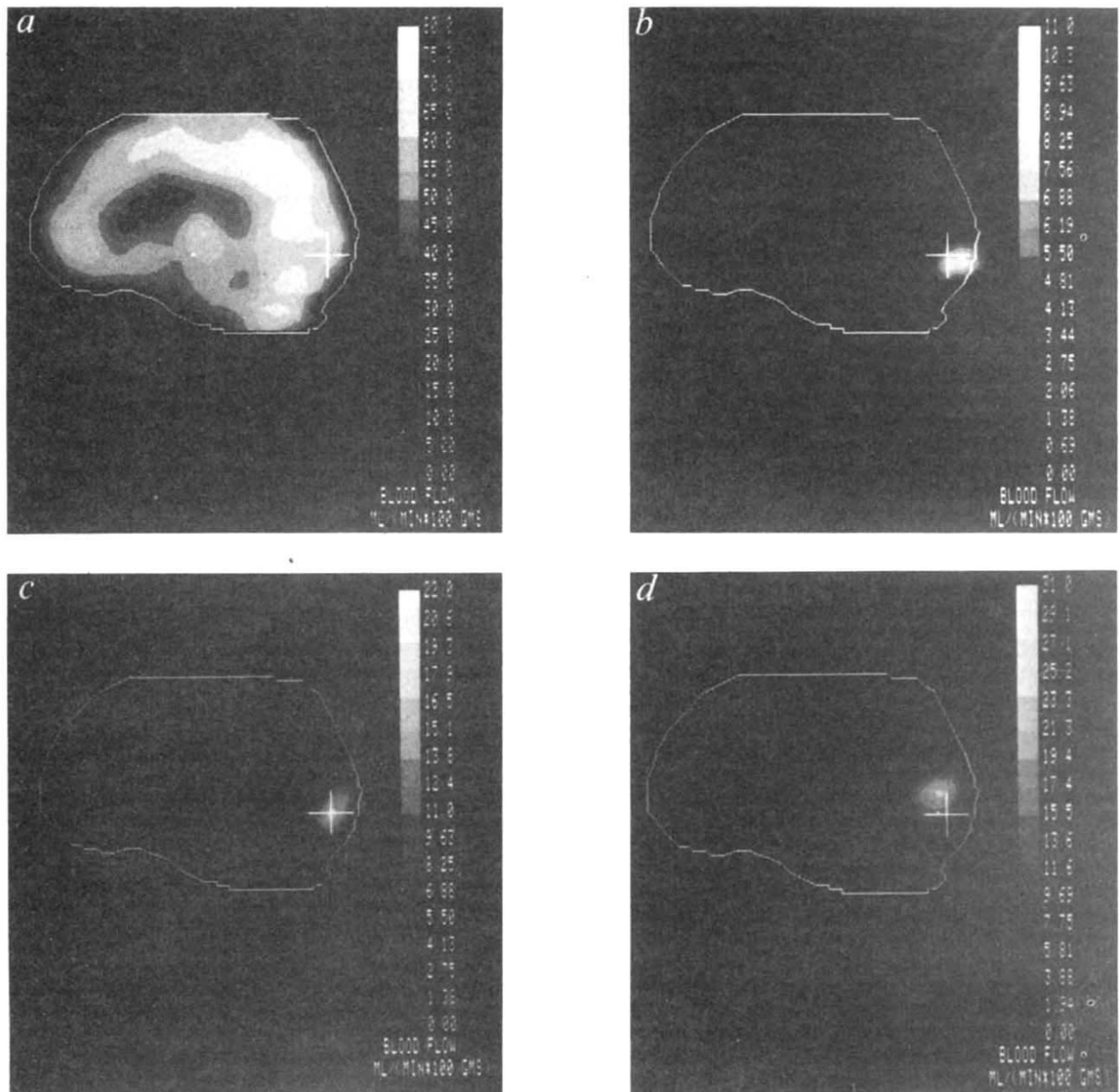


Fig. 3 All images are midsagittal sections formed by summing the images of all six subjects within a standardized stereotactic coordinate space⁹. Anterior is to the left. The brain boundary was obtained from image A and is positioned identically in all four images. The horizontal fiducial lies on the line connecting the anterior and posterior commissures (bicommissural or AC-PC line⁹). The vertical fiducial lies 6.2 cm posterior to the centre of the bicommissural line. Both fiducials are 2.0 cm long. Units of blood flow are $\text{ml g}^{-1} \text{min}^{-1}$. *a*, Resting-state image of cerebral blood flow (CBF). *b*, Macular response focus ($0.1\text{--}1.5^\circ$ stimulus) in a subtraction image format. The response lies most posterior and inferior, at the occipital pole. *c*, Perimacular response focus ($1.5\text{--}5.5^\circ$ stimulus) in subtraction image format, intermediate in location between the macular and peripheral responses. *d*, Peripheral response focus ($5.5\text{--}15.5^\circ$ stimulus) in subtraction image format, lying anterior and superior to the two other responses.

here yielded an orderly, continuous, retinotopic map in agreement with the order of retinal projections to visual cortex observed in non-human primates^{20,21} and expected by homology in man. The anatomical precision of this map exceeds those previously attained in man. These observations also have provided the first direct, non-invasive measurements of cortical magnification factor in humans.

Computer simulations (to be reported separately) were performed to define further the properties of this imaging strategy. Three findings bear mention. First, the accuracy with which a focal CBF change is localized increases with the intensity of the CBF response. Second, localization accuracy of $0.5\text{--}1.5\text{ mm}$ can be attained with an image resolution of 18 mm (FWHM) and focal CBF change of only 20–30% (comparable to those

observed in this study). Finally, the effective spatial resolution achieved with this technique should increase with the spatial resolution (FWHM) of the tomograph employed. As PET instrumentation evolves, therefore, the precision achieved with this mapping strategy should continue to improve.

Applications of this strategy for high-resolution functional brain mapping potentially are quite broad. In principle, any brain region undergoing increased neuronal work and, thereby, increased regional CBF during task performance can be mapped. Mapping precision, however, will vary with the intensity and focality of the task's induced CBF change, both of which will be strongly affected by task design^{4,5,8}. Our pilot application of this technique mapped primary visual cortex. Similar studies of primary somatosensory and auditory cortices

Table 2 Magnification factor in human striate cortex

Comparison	Macula to perimacula	Macula to periphery	Perimacula to periphery
Response shift (mm)	9.4	15.6	6.6
Stimulus shift (degrees)	2.8	9.8	7.0
Mean eccentricity (degrees)	2.1	5.6	7.0
Magnification factor (mm per degree)	3.4	1.6	0.9

These values were calculated from the mean eccentricity of each stimulus pattern (macula = 0.7°, perimacula = 3.5°, periphery = 10.5°) and the mean response locale for each stimulus locale as given in Table 1. Degrees indicate degrees of eccentricity from the fixation point. Magnification factors are expressed in millimetres of striate cortex per degree of eccentricity of retinal visual field¹⁰⁻¹².

and higher-order cortical zones are under way. Detailed mapping of brain areas participating in such uniquely human activities as language, cognition and emotion should follow in due course and hold still greater interest and potential.

Received 10 January; accepted 11 August 1986.

1. Yamamoto, M., Ficke, D. C. & Ter-Pogossian, M. M. *IEEE Trans. nucl. Sci.* **29**, 529-533 (1982).
2. Lahti, B. P. *Signals, Systems, and Communication*, 437-441 (New York, 1965).
3. Westheimer, G. *Invest. ophthalm. vis. Sci.* **18**, 893-912 (1979).
4. Fox, P. T. & Raichle, M. E. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1140-1144 (1986).
5. Fox, P. T. & Raichle, M. E. *J. Neurophysiol.* **51**, 1109-1120 (1984).
6. Herscovitch, P., Markham, J. & Raichle, M. E. *J. nucl. Med.* **24**, 782-789 (1983).
7. Raichle, M. E., Martin, W. R. W., Herscovitch, P., Mintun, M. A. & Markham, J. *J. nucl. Med.* **24**, 790-798 (1983).
8. Fox, P. T., Fox, J. M., Raichle, M. E. & Burde, R. M. *J. Neurophysiol.* **54**, 348-369 (1985).
9. Fox, P. T., Perlmutter, J. S. & Raichle, M. E. *J. Comput. Assist. Tomogr.* **9**, 141-153 (1985).
10. Daniel, P. M. & Whitteridge, D. *J. Physiol., Lond.* **159**, 203-221 (1961).
11. Cowey, A. & Rolls, E. T. *Expl Brain Res.* **21**, 447-454 (1974).
12. Rovamo, J. & Virsu, V. *Expl Brain Res.* **37**, 495-510 (1979).
13. Holmes, G. *Br. J. Ophthalm.* **2**, 353-384 (1918).
14. Spalding, J. M. D. *J. Neurol. Neurosurg. Psychiat.* **15**, 169-183 (1952).
15. Teuber, H., Battersby, W. & Bender, M. *Visual Field Deficits after Penetrating Missile Wounds* (Harvard University Press, Cambridge, 1960).
16. McAuley, D. L. & Ross-Russell, R. W. *J. Neurol. Neurosurg. Psychiat.* **42**, 298-311 (1979).
17. Brindley, G. S. in *Handbook of Sensory Physiology* **7** (3B) (ed. Jung, R.) 583-594 (Springer, Berlin, 1973).
18. Dobelle, W. H. & Mladejovsky, M. G. *J. Physiol., Lond.* **243**, 553-576 (1974).
19. MacLin, E., Okada, Y. C., Kaufman, L. & Williamson, S. J. *Nuov. Cim.* **2**, 410-419 (1983).
20. Van Essen, D. C., Newsome, W. T. & Maunsell, J. H. R. *Vision Res.* **24**, 429-448 (1984).
21. Talbot, S. A. & Marshall, W. H. *Am. J. Ophthalm.* **24**, 1255 (1941).

Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis

Helen V. New & Anne W. Mudge

MRC Neuroimmunology Programme, Department of Zoology, University College London, Gower Street, London WC1E 6BT, UK

Innervation of muscle by motoneurons induces the development of a characteristic, high density cluster of acetylcholine receptors (AChRs) at the neuromuscular junction¹⁻⁴. Studies *in vitro* show that the accumulation of AChRs at nerve-muscle contacts results from both increased insertion of new AChRs into the muscle plasma membrane beneath nerve terminals⁵ and redistribution of pre-existing AChRs⁶⁻⁷; these two modes of AChR accumulation may be separately controlled since factors have been identified that influence AChR redistribution but not synthesis^{8,9}. Although many aspects of muscle development are regulated by nerve-dependent muscle activity¹⁰⁻¹⁶, junctional AChR clusters still develop when neuromuscular transmission is blocked by either curare or α -bungarotoxin^{1,5,6,17}, suggesting that their formation is mediated

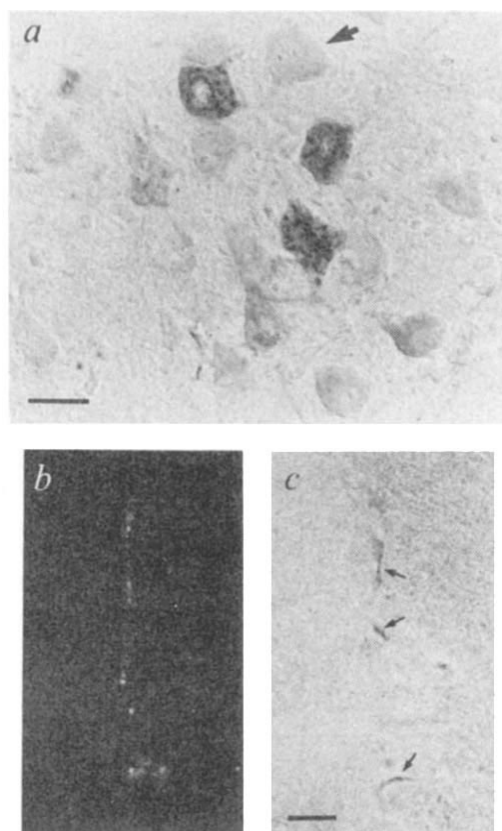


Fig. 1 Immunohistochemical localization of CGRP-I in embryonic chick motoneurons. *a*, Granular CGRP immunoreactivity in a subpopulation of motoneurone cell bodies at E17. The arrow points to an unstained motoneurone. Bar = 30 μ m. *b*, CGRP immunoreactivity in a section of E14 leg muscle. *c*, AChE staining of the same field as in *b*. Arrows point to neuromuscular junctions. Bar = 10 μ m.

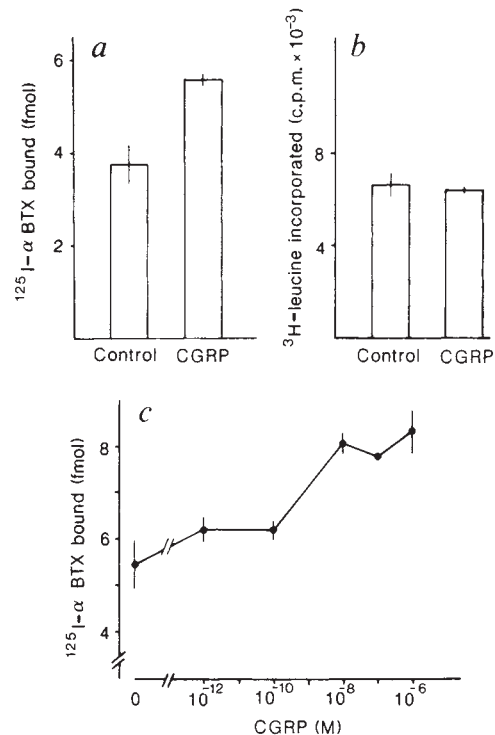
Methods. Cryostat sections of tissue fixed in 4% paraformaldehyde (pfa) were processed for staining by the peroxidase anti-peroxidase method using 3,3'-diaminobenzidine tetrahydrochloride (Poly-sciences) as described previously²². Rabbit antiserum to CGRP (Cambridge Research Biochemicals; lot CGR-37) was used at 1:400. Staining was abolished in control serial sections incubated with antiserum together with 10^{-7} M CGRP (Sigma). For double-labelling of sections with AChE and CGRP, sections were first stained for AChE³⁴, washed and then incubated with anti-CGRP antibody overnight at 4°C. The second antibody was sheep anti-rabbit-immunoglobulin conjugated to fluorescein isothiocyanate (Wellcome), at 1:100. Sections mounted in glycerol with DABCO³⁵; viewed with a Zeiss microscope equipped with either epifluorescence (*b*) or differential interference contrast optics (*c*).

by nerve-derived trophic factors other than activity. A molecule immunologically related to calcitonin gene-related peptide (CGRP-I) has been found in motoneurons in a variety of mammals including man^{18,19}. Here we provide indirect evidence that CGRP-I may be a motoneurone-derived trophic factor that increases AChR synthesis at vertebrate neuromuscular junctions.

A rabbit antiserum to CGRP was used to stain cryostat sections from the lumbosacral region of developing chicks by the peroxidase anti-peroxidase method. Many large cells in the ventral horn of the spinal cord showed granular cytoplasmic staining (Fig. 1*a*); from their size and position in the lateral motor column (LMC) these cells were identified as motoneurons. CGRP-I was present in a few cells of the chick lumbosacral LMC at embryonic day 6 (E6), and was in approximately half the motoneurons throughout the rostrocaudal axis from E7.5 onward. Although a subpopulation of motoneurons was strongly CGRP-immunoreactive throughout development,

Fig. 2 Effect of 10^{-7} M CGRP on cell-surface AChR number (125 I- α BTX binding sites) (a) and protein synthesis (b) in cultured chick myotubes. c, Dose response for effect of CGRP on AChR number. All points in a and b are from sister cultures. Bars indicate $\pm$ s.e.m.; $n=5$ (a, b); $n=4$ (c). The data in a and c have been corrected for nonspecific 125 I- α BTX binding, which was approximately 5%.

Methods. Muscle cells from E11 chick pectoral and thigh muscle were dissociated in Ca^{2+} -, Mg^{2+} -free, HEPES-buffered, balanced salt solution (BSS) for 30 min at 37°C and plated in 96-well tissue-culture trays (Falcon) coated with 1% gelatin at 5,000 cells per well. Growth medium was Dulbecco's modified Eagle medium (DMEM) (Gibco) with 10% horse serum, 5% E11 chick embryo extract (CEE), plus 50 units ml^{-1} penicillin, 50 units ml^{-1} streptomycin and 4 mM glutamine. After 2 days, the CEE was reduced to 2%. For these experiments, CGRP was added for 24 h on day 2. In experiments where CGRP was added for longer periods, cells were fed with 5×10^{-6} M cytosine arabinoside (Sigma) from day 2 to 3 to reduce the number of fibroblasts. The number of AChRs was measured by binding α BTX (Boehringer) labelled with Na^{125}I (Amersham) by the chloramine-T method³⁶. The cells were incubated at 37°C for 60 min in 5×10^{-9} M ^{125}I - α BTX (specific activity $120\text{--}170$ Ci mmol^{-1}) in DMEM plus 1% bovine serum albumin, then washed four times for a total of 15 min in phosphate-buffered saline plus 4% calf serum. The cells were collected with 0.25% trypsin (Sigma) and counted in a gamma counter (Nuclear Enterprises 1600). Nonspecific binding of the ^{125}I - α BTX was measured by incubating some wells in the presence of 10^{-3} M carbachol (Sigma). For measurement of protein synthesis, $1 \mu\text{Ci}$ ^3H -leucine (specific activity 170 Ci mmol^{-1} , Amersham) was added to each well for 4 h before sister cultures were either used for binding with ^{125}I - α BTX as above, or extracting protein. For the latter, cells were collected in 10 mM Tris, 1% NP40, 1 mM EDTA, protein was precipitated onto glass filters (Whatman) by trichloroacetic acid and the filters counted in a scintillation counter (LKB). This experiment was repeated four times with similar results.



some were completely negative in our assay and others were only weakly stained. It is not known which muscle masses the CGRP-negative motoneurons innervate or whether these neurones contain other peptides. Although some ciliary neurones innervate striated muscle *in vivo*, we could not detect CGRP-I in these neurones from E8-E10. The time course of appearance of CGRP-I in motoneurons during chick development paralleled the time course of formation of neuromuscular synapses. Functional neuromuscular synapses have been detected as early as E5 in the chick limb²⁰, but it is not until E6.5 that motoneurons ramify widely within muscle masses²¹. Later in development, from E11 onward, a large increase in the number of high density AChR clusters in the posterior latissimus dorsi muscle has been described¹⁷, probably due to the formation of synapses on new myotubes. In addition, we found that CGRP-I appeared in rat motoneurons on postnatal day 3 (not shown).

To determine whether CGRP-I was present in embryonic motoneurone terminals as well as in their cell bodies, leg muscles from E14 embryos were stained for CGRP, and at the same time with acetylcholinesterase (AChE) to identify neuromuscular junctions. As shown in Fig. 1b and c, CGRP-I was present in nerve fibres at a subpopulation of AChE-stained sites. CGRP-I was also seen in nerve fibres in blood vessel walls (not shown); these were probably sensory fibres as we have previously shown that CGRP-I is present in small sensory neurones in the chick peripheral nervous system from E10 onward²².

Because CGRP-I is present in motoneurone terminals, we tested the possibility that it is involved in the nerve-dependent accumulation of junctional AChRs. Muscle cells from E11 chicks were grown in culture with and without CGRP, and the number of AChRs present on myotubes was assessed by the binding of ^{125}I - α -bungarotoxin (^{125}I - α BTX). When 10^{-7} M CGRP was added to 2-day-old myotubes for 24 h, there was a consistent ($\sim 40\%$) increase in the total number of AChRs (Fig. 2a). The increase was not part of a general effect of CGRP on

protein synthesis: in experiments where CGRP increased AChR number by 50%, there was no effect on the rate of incorporation of ^3H -leucine into total protein (Fig. 2a,b). The effect on AChR number was seen at low concentrations of CGRP, and was saturable (Fig. 2c); the half maximal dose was approximately 10^{-9} M, although this may be an overestimate since the stability of CGRP in culture is not known. Increasing the time of exposure to CGRP did not increase the effect. Similar effects on AChR number were seen if CGRP was added to older cultures or to cultures that had been treated with 5×10^{-6} M cytosine arabinoside to kill dividing cells (not shown), suggesting that the CGRP effect was not due simply to an increase in muscle cell numbers. CGRP also increased AChR number in 4-day-old cultures of neonatal rat myotubes grown by standard procedures (not shown).

The increase in cell-surface AChRs in the presence of CGRP could reflect either an increase in the rate of insertion of new receptors into the plasma membrane or a decrease in the rate of degradation of pre-existing ones, or both. The effect of CGRP on insertion of AChRs was studied by blocking pre-existing AChRs on 3-day-old myotubes with unlabelled α BTX. After washing, the cells were incubated with or without CGRP and the number of new ^{125}I - α BTX binding sites measured at various times. While there was a linear increase in the number of new AChRs inserted in control cultures, 4 h after CGRP addition, the rate of receptor insertion was increased by about 45% in CGRP-treated cultures (Fig. 3a), and this increase continued for the next 7 h. In addition, when cells were pretreated with CGRP for 24 h, an increase in insertion rate could still be measured. The CGRP effect must have been due to an increase in the rate of synthesis of new receptors rather than in the rate of incorporation of pre-existing intracellular ones since AChRs are inserted into the plasma membrane within 3.5 hours of synthesis²³.

The rate of degradation of AChRs was estimated by prelabel-

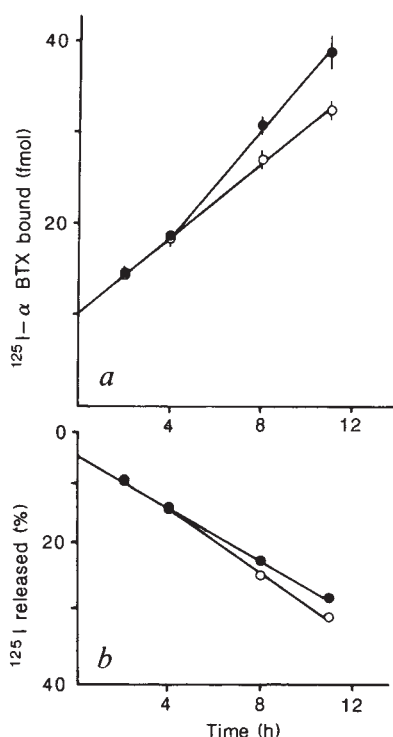


Fig. 3 Effect of 10^{-8} M CGRP on AChR insertion into the plasma membrane of cultured chick myotubes (a) and AChR degradation in sister cultures (b). Bars indicate $\pm$ s.e.m.; $n=4$. $\circ$, control; $\bullet$, CGRP.

Methods. Insertion and degradation experiments were done as previously described²⁵. To measure insertion of new AChRs into the plasma membrane, E11 chick myotubes in culture for 2 days (plated at 10,000 per well in 24-well tissue culture trays as for Fig. 2) were incubated in 1.25×10^{-8} M unlabelled α -BTX for 30 min at 37 °C, washed four times in a total of 2 litres of HEPES-buffered BSS over 30 min, then incubated in growth medium with or without 10^{-8} M CGRP. At various times after the addition of CGRP, newly inserted AChR were measured by incubating cells with 1.25×10^{-8} M ^{125}I - α -BTX for 30 min, followed by washing and collecting as for Fig. 2. Nonspecific binding was not measured in these experiments. To measure the degradation of pre-existing AChR, cells were first labelled with ^{125}I - α -BTX (1.25×10^{-8} M, specific activity 170 Ci mmol⁻¹), then washed as above and incubated for various times with or without the addition of 10^{-8} M CGRP. Both supernatant and cells (collected as in Fig. 2) were counted, and AChR degradation was expressed as the percentage of counts in the supernatant compared to the total counts in the supernatant plus cells. Other similar results were obtained in two separate experiments.

ling the receptors with ^{125}I - α -BTX and measuring the number of counts released into the medium at later times as a proportion of the original total²⁴. Four hours after the addition of CGRP there was a small but significant decrease in the rate of release of label compared to controls (Fig. 3b). However, this apparent decrease in degradation rate is negligible when the data are corrected for the greater dilution of prelabelled AChRs by insertion of new receptors in CGRP-treated cultures compared with controls. Therefore, the increased accumulation of AChRs in the presence of CGRP predominantly reflected an increase in the rate of synthesis.

To study the effect of CGRP on AChR distribution, cells were treated with CGRP for 24 h, AChR were labelled with ^{125}I - α -BTX, and the cultures processed for autoradiography²⁵. In one experiment, where there was a high number of clusters there was a statistically significant increase in the number of AChR clusters in CGRP-treated cultures compared to controls, which

paralleled the increase in AChR number in sister cultures. However, we could not determine whether this increase was due to an independent effect on AChR clustering, or simply to the increase in AChR number, although we think that the latter is the more likely.

Our results indicate that CGRP increases the rate of synthesis and insertion of AChRs into the plasma membrane of cultured myotubes without increasing general protein synthesis. In contrast to CGRP, a protein identified in detergent extracts of *Torpedo* electric organ⁹ (and shown by antibody staining to be at the *Torpedo* neuromuscular junction²⁶) produces a dramatic increase in the number of high-density AChR clusters on chick myotubes in the absence of an effect on AChR number. On the other hand, an ascorbate-like molecule, which has been shown to be released from spinal cord neurone terminals²⁷, induces AChR clustering on cultured primary muscle cells²⁸, and both AChR clustering and synthesis on muscle cell lines²⁹. Fischbach and colleagues have identified both high³⁰ and low relative molecular mass³¹ components of brain extracts that increase clustering and synthesis of AChRs on cultured chick myotubes³²; the effect of the low relative molecular mass molecules on AChR synthesis after 24 h was quantitatively similar to the effect of CGRP in our experiments³¹.

Because CGRP-I is present in motoneurons during the period of neuromuscular junction formation and CGRP is able to increase AChR synthesis in myotubes *in vitro*, CGRP-I must be a strong candidate for a muscle trophic factor released from motoneurone terminals *in vivo*: by stimulating a local increase in AChR synthesis, it could be involved in the formation of junctional AChR clusters. It will be interesting to determine whether CGRP increases ongoing AChR messenger RNA transcription or translation or both, or whether it induces the production of a new AChR mRNA specific to the innervated state³³.

We thank Martin Raff and our other colleagues in the MRC Neuroimmunology Programme for their many helpful comments on the manuscript, and D. Oliveira for his assistance with the statistics programme. H.V.N. was supported by an MRC Research Studentship.

Received 11 June; accepted 19 July 1986.

- Anderson, M. J., Cohen, M. W. & Zorychta, E. *J. Physiol., Lond.* **268**, 731-756 (1977).
- Frank, E. & Fischbach, G. D. *J. Cell Biol.* **83**, 143-158 (1979).
- Harris, A. J. *Phil. Trans. R. Soc. B293*, 287-314 (1981).
- Chow, I. & Cohen, M. W. *J. Physiol., Lond.* **339**, 553-571 (1983).
- Role, L. W., Matossian, V. R., O'Brien, R. J. & Fischbach, G. D. *J. Neurosci.* **5**, 2197-2204 (1985).
- Anderson, M. J. & Cohen, M. W. *J. Physiol., Lond.* **268**, 757-773 (1977).
- Ziskind-Conhaim, L., Geffen, I. & Hall, Z. W. *J. Neurosci.* **4**, 2346-2349 (1984).
- Christian, C. N. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 4011-4015 (1978).
- Nitkin, R. M., Wallace, B. G., Spira, M. E., Godfrey, E. W. & McMahan, U. J. *Cold Spring Harb. Symp. quant. Biol.* **48**, 653-665 (1983).
- Lomo, T. & Rosenthal, J. *J. Physiol., Lond.* **221**, 493-513 (1972).
- Burden, S. *Dev. Biol.* **57**, 317-329 (1977).
- Rubin, L. L., Schuetz, S. M., Weill, C. L. & Fischbach, G. D. *Nature* **283**, 264-267 (1980).
- Srihari, T. & Vrbová, G. *J. Neurocytol.* **7**, 529-540 (1978).
- Lomo, T., Westgaard, R. H. & Dahl, H. A. *Phil. Trans. R. Soc. B187*, 99-103 (1974).
- Pette, D., Müller, W., Leisner, E. & Vrbová, G. *Pflügers Arch. ges. Physiol.* **364**, 103-112 (1976).
- Harris, A. J. *Phil. Trans. R. Soc. B293*, 257-277 (1981).
- Beitz, H., Bourgeois, J.-P. & Changeaux, J.-P. *J. Physiol., Lond.* **302**, 197-218 (1980).
- Rosenfeld, M. G. *et al. Nature* **304**, 129-135 (1983).
- Gibson, S. J. *et al. J. Neurosci.* **4**, 3101-3111 (1984).
- Landmesser, L. *J. Physiol., Lond.* **284**, 391-414 (1978).
- Tosney, K. W. & Landmesser, L. *T. J. Neurosci.* **5**, 2336-2344 (1985).
- New, H. V. & Mudge, A. *Dev. Biol.* **116**, 337-346 (1986).
- Devreotes, P. N., Gardner, J. M. & Fambrough, D. M. *365-373* (1977).
- Devreotes, P. N. & Fambrough, D. M. *J. Cell Biol.* **65**, 335-358 (1975).
- French-Constant, C. & Raff, M. C. *Nature* **319**, 499-502 (1986).
- Fallon, J. R., Nitkin, R. M., Reist, N. E., Wallace, B. G. & McMahan, U. J. *Nature* **315**, 571-574 (1985).
- Kalchauer, C., Bachar, E., Duxin, D. & Vogel, Z. *Neurosci. Lett.* **58**, 219-224 (1985).
- Kalchauer, C., Vogel, Z. & Duxin, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3077-3081 (1982).
- Knaack, D., Shen, I., Salpeter, M. M. & Podleski, T. R. *J. Cell Biol.* **102**, 795-802 (1986).
- Udin, T. & Fischbach, G. D. *J. Cell Biol.* **103**, 493-507 (1986).
- Buc-Caron, M.-H., Nystrom, P. & Fischbach, G. D. *Dev. Biol.* **95**, 378-386 (1983).
- Jessell, T. M., Siegel, R. E. & Fischbach, G. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5397-5401 (1979).
- Mishina, M. *et al. Nature* **321**, 406-411 (1986).
- Karnovsky, M. J. & Roots, L. J. *Histochem. Cytochem.* **12**, 219-221 (1964).
- Johnson, G. D. *et al. J. Immun. Meth.* **55**, 231-242 (1982).
- Brookes, J. P. & Hall, Z. W. *Biochemistry* **14**, 2092-2106 (1975).

cGMP-dependent protein kinase enhances Ca^{2+} current and potentiates the serotonin-induced Ca^{2+} current increase in snail neurones

Danièle Paupardin-Tritsch*, Constance Hammond*,
Hersch M. Gerschenfeld*, Angus C. Nairn†
& P. Greengard†

* Laboratoire de Neurobiologie, Ecole Normale Supérieure,
46 Rue d'Ulm, 75230 Paris Cedex 05, France

† Rockefeller University, 1230 York Avenue, New York,
New York 10021-6399, USA

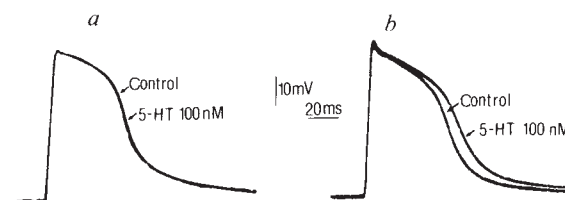


Fig. 1 Effect of cGMP-PK on the Ca^{2+} -dependent action potential recorded from a D6 neurone bathed in tetraethylammonium (TEA)-saline. *a*, The application of 100 nM serotonin (5-HT) evokes no alteration in the duration of the action potential. The trace corresponds to the superimposed recordings of the action potential in the absence and presence of 100 nM serotonin. *b*, After washing out serotonin, a new control action potential is recorded and then after repetitive pressure injections of cGMP-PK (5 pulses of 50 ms duration, pressure 3 mm Hg), 100 nM serotonin is applied, increasing the duration of the action potential. Results similar to those shown here were obtained in each of six experiments.

Methods. The isolated and desheathed abdomino-visceral ganglionic mass of the snail *H. aspersa* was bathed in saline of the following composition (in mM): NaCl 120, KCl 2.5, CaCl_2 6, MgCl_2 3.5, HEPES 10 (pH 7.4). To unmask the Ca^{2+} -dependent component of the action potential, the cells were bathed in saline in which 30 mM NaCl was replaced with 30 mM TEA-Cl (TEA-saline). Serotonin-creatinine sulphate was dissolved in the saline. The identified snail ventral neurones were impaled with two micro-electrodes connected to a current/voltage clamp system. To inject cGMP-PK intracellularly two techniques were used: (1) pressure injection using siliconized micropipettes which had tips of relatively small diameter (1–2 μm) and in which the application of a pressure pulse (2 mm Hg, 10 ms) did not cause the appearance of a fluid drop at the tip, under microscopic examination; (2) 'release' injection (without applying pressure) using siliconized micropipettes which had tips of larger diameter. A short pressure pulse (2 mm Hg, 10 ms) applied to these micropipettes evoked the expulsion of a large fluid drop ('leaky' micropipettes). cGMP-PK was purified to homogeneity from bovine lung by the method of Walter *et al.*¹⁷. Using histone f_2b as a substrate¹⁷, the enzyme had a specific activity of 3 μmol per min per mg. It was stored at -20°C in 20 mM Tris-HCl (pH 7.5)/1 mM EDTA/5 mM 2-mercaptoethanol/50% glycerol, and dialysed against 10 mM HEPES (pH 7.4)/100 mM KCl for use in the injection studies.

Protein phosphorylation catalysed by cyclic AMP-dependent^{1,2}, Ca^{2+} /calmodulin-dependent³ and Ca^{2+} /diacylglycerol-dependent⁴ protein kinases is important both in the modulation of synaptic transmission and in the regulation of neuronal membrane permeability (for reviews see refs 5–7). However, there has previously been no evidence for the involvement of cyclic GMP-dependent protein kinase (cGMP-PK) in the regulation of neuronal function. Serotonin induces an increase of Ca^{2+} current in a group of identified ventral neurones of the snail *Helix aspersa*⁸. This effect is probably mediated by cGMP because it is mimicked by the intracellular injection of cGMP or the application of zaprinast⁹, an inhibitor of cGMP-dependent phosphodiesterase⁹. We have now found that the effect of either serotonin or zaprinast on the Ca^{2+} current is potentiated by the intracellular injection of cGMP-PK. Moreover, the intracellular injection of activated cGMP-PK (cGMP-PK + 1 μM cGMP) greatly enhances the Ca^{2+} current of the identified ventral neurones seen in the absence of serotonin. These results indicate that cGMP-PK has a physiological role in the control of the membrane permeability of these neurones.

We first examined the effect of cGMP-PK on the serotonin-induced broadening of the Ca^{2+} -dependent plateau of the somatic action potential of identified snail ventral neurones (cells D6, D7). The neurones ($n=6$) were impaled with two microelectrodes connected both to a current/voltage clamp device and to a micropipette filled with cGMP-PK. Before protein kinase injections, the application of a low concentration (100 nM) of serotonin had no effect on the duration of the Ca^{2+} -dependent spike (Fig. 1*a*). Repetitive intracellular pressure injections of cGMP-PK, by themselves, were also without effect. However, after injection of cGMP-PK, 100 nM serotonin produced an increase in the duration of the Ca^{2+} -dependent action potential (Fig. 1*b*).

In a second series of experiments, we investigated the effect of cGMP-PK on the inward current through the Ca^{2+} channels of the voltage-clamped ventral neurones. In each of eight neurones tested, the effect of low concentrations of serotonin on the inward current was potentiated by prior injection of cGMP-PK. The application of 100 nM serotonin elicited a small increase in the amplitude of the inward current in control conditions (Fig. 2*a*i), but when applied after repetitive intracellular injection of cGMP-PK the same serotonin concentration caused marked enhancement of the inward current (Fig. 2*a*, ii). Furthermore, in three other neurones we found that pressure injections of cGMP-PK lowered the serotonin concentration required to evoke a detectable change in the inward current (from 25 nM to 1 nM).

In each of five neurones, the effect of low concentrations of zaprinast on the inward current was also potentiated by cGMP-PK injection. Whereas 1 μM zaprinast had no effect on the inward current under control conditions (Fig. 2*b*i), when it was applied after repetitive intracellular injections of cGMP-PK this concentration caused a marked enhancement of the inward current (Fig. 2*b*, ii). In each of six neurones, repetitive intracel-

lular pressure injections of cGMP-PK alone evoked little or no enhancement of the inward current (not shown).

We then investigated how the inward current of the ventral neurones was affected by the injection of cGMP-PK that was pre-activated by the addition of 1 μM cGMP to the enzyme solution. In three of six neurones in which the activated cGMP-PK was pressure injected into the cells, the enzyme evoked an increase in the amplitude of the inward current (Fig. 3*a*). In each of eight experiments in which the activated cGMP-PK was released into the neurones from large-tip ('leaky') micropipettes (release injection), the effect of the enzyme on the inward current was much larger and more consistent. For example, in the experiment shown in Fig. 3*b*, the control inward current was recorded before impaling the cell with a leaky micropipette containing the cGMP-PK (Fig. 3*b*, control). When the leaky micropipette containing the enzyme was introduced into the neurone and the activated cGMP-PK released into the cell, it more than doubled the amplitude of the inward current (Fig. 3*b*, activated cGMP-PK). In almost all experiments in which activated cGMP-PK was injected into cells from leaky micropipettes, the inward current attained amplitudes of at least 400 nA, that is, 200–300% of control. In contrast, serotonin has never evoked an increase in Ca^{2+} current of larger than 25–30% of the control value, presumably because the endogenous levels of cGMP-PK are rate-limiting in mediating serotonin effects. Moreover, in some ventral neurones impaled with the leaky enzyme pipettes, the inward current increased continuously until the cell suddenly died.

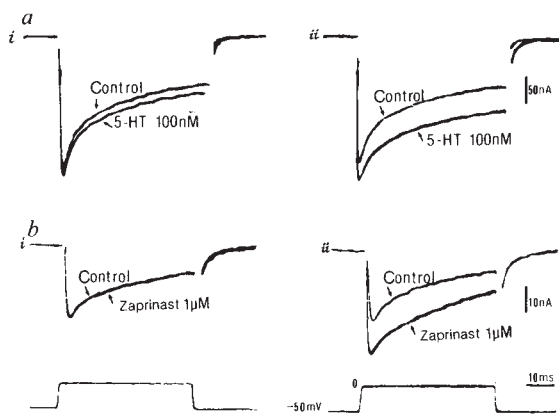


Fig. 2 Effect of serotonin (ai,ii) and zaprinast (bi,ii) on the inward current recorded from a voltage-clamped D6 neurone before and after cGMP-PK injection. *ai*, Serotonin (100 nM) elicits a small increase in the amplitude of the inward current. *a*ii, After washing out serotonin, repetitive intracellular pressure injections of non-activated cGMP-PK are performed (8 pulses of 80 ms duration, pressure 2.5 mm Hg) and immediately after obtaining a new control recording, 100 nM serotonin is applied. The Ca^{2+} current and tail current are both increased. *bi*, Inward current recorded from a different D6 neurone. Zaprinast (1 μM) has no effect on the inward current. The trace corresponds to superimposed recordings of the inward current in the presence and absence of zaprinast. *b*ii, After washing out zaprinast, repetitive intracellular injections of cGMP-PK (6 pulses of 50 ms duration, pressure 3 mmHg) are performed and, after obtaining a new control recording, 1 μM zaprinast now evokes a large increase in the amplitude of the inward current. **Methods.** To isolate the inward current through the Ca^{2+} channels, the neurones were bathed in saline containing both 1 μM tetrodotoxin (TTX) and 30 mM TEA and in which Ca^{2+} was replaced with Ba^{2+} (TTX/TEA/ Ba^{2+} -saline)^{18,19}. To record the inward current in this figure and in Fig. 3, the neurones were held at -50 mV and depolarized to 0 mV for 60 ms. In *a* the cells were repolarized to -75 mV at the end of the pulse.

One set of control experiments ($n=7$) consisted of either pressure injection or release injection of cGMP-PK which had been totally inactivated by heating at 56 °C for 10 min. The heat-inactivated enzyme never potentiated the effects of either 1–10 μM serotonin or 1–20 μM zaprinast⁹ on the inward current (not shown). In another set of control experiments ($n=4$), when 1 μM cGMP was pressure injected or released from leaky micropipettes inside the neurones, the inward current was not

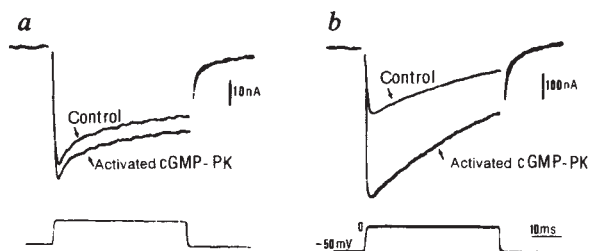


Fig. 3 Effect of activated cGMP-PK on the inward current through the Ca^{2+} channels, in the absence of serotonin, using two different intracellular injection techniques. *a*, Recordings were obtained from a voltage-clamped D6 neurone bathed in TTX/TEA/ Ba^{2+} -saline before and after pressure injection of activated cGMP-PK (cGMP-PK + 1 μM cGMP; 10 pulses of 40 ms duration, pressure 3 mm Hg) from a small-tip-diameter micropipette. *b*, A control recording was obtained from another D6 neurone bathed in TTX/TEA/ Ba^{2+} -saline; the cell was then bathed in normal saline and impaled with a leaky micropipette containing activated cGMP-PK; the preparation was bathed again in TTX/TEA/ Ba^{2+} -saline and the inward current measured.

increased. Previous experiments showing that cGMP does increase the inward current⁸ used pressure injection micropipettes containing higher cGMP concentrations (0.1 M).

We propose the following scheme for the mechanism by which serotonin modulates voltage-sensitive Ca^{2+} channels in identified ventral neurones of *H. aspersa*. By activating a receptor on the plasma membrane, serotonin causes an increase in the intracellular concentration of cGMP leading to the activation of cGMP-PK, which in turn causes phosphorylation either of the Ca^{2+} channel or of a regulatory protein closely associated with the Ca^{2+} channel.

Several results support this scheme. First, serotonin and zaprinast both increase the inward current in identified ventral neurones⁸. Serotonin, in the presence of low concentrations of zaprinast, and zaprinast at higher concentrations, both raise the level of cGMP in pools of isolated ventral neurones (D.P.-T., C.H. and J. Grassi, unpublished). Second, intracellular injection of cGMP mimics the effect of serotonin and zaprinast in increasing the Ca^{2+} current⁸. Third, the injection of cGMP-PK potentiates the effect of serotonin and zaprinast in increasing the Ca^{2+} current (present results). Finally, the injection of activated cGMP-PK (cGMP-PK + 1 μM cGMP) increases the Ca^{2+} current seen in the absence of serotonin (present results).

The injection of cGMP-PK which had not been activated by prior addition of cGMP had little or no effect on the Ca^{2+} current seen in the absence of serotonin. This may be due to the low and variable cGMP content observed in the identified ventral neurones (D.P.-T., C.H. and J. Grassi, unpublished) which is presumably not high enough to activate the injected cGMP-PK. The activated form of cGMP-PK used in the present study is analogous to the catalytic subunit of cAMP-dependent protein kinase (cAMP-PK), the active form of that enzyme which was injected into cells in previous studies. In those studies, the injection of the catalytic subunit of cAMP-PK was found to mimic the effect of neurotransmitters that raise cAMP levels, in the absence of the relevant neurotransmitters or cAMP^{1,2,10–12}.

Cyclic GMP has been reported to increase the photosensitive cationic conductance of isolated inside-out patches of the plasma membrane of vertebrate retinal rod outer segments^{13,14} and, more recently, the cation-selective channels activated by cGMP in these excised membrane patches have been characterized^{15,16}. The mechanism by which cGMP opens these channels does not seem to involve protein phosphorylation. In contrast, the present work provides direct evidence that cGMP increases the Ca^{2+} conductance of snail neurones by activating cGMP-PK.

These results, together with other recent studies, show that Ca^{2+} currents can be modulated by at least three classes of protein kinases. The Ca^{2+} channels of cardiac muscle cells are modulated by a cAMP-dependent protein kinase^{10,11}, Ca^{2+} current of *Aplysia* bag cells can be modulated by Ca^{2+} /diacylglycerol-dependent protein kinase⁴ and, as shown in this study, the Ca^{2+} current of identified ventral neurones of *Helix aspersa* is modulated by cGMP-PK.

This work was supported by grants from the CNRS (UA 295 and ATP Pharmacologie Moléculaire de la Neurotransmission) and from the Université P. et M. Curie, Paris, to H.M.G. and D.P.-T., and by the USPHS grant MH40899 to A.C.N. and P.G.

Received 6 May; accepted 12 August 1986.

- Kaczmarek, L. K. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7487–7491 (1980).
- Castellucci, V. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7492–7496 (1980).
- Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3035–3039 (1985).
- DeRiemer, S. A., Strong, J. A., Albert, K. A., Greengard, P. & Kaczmarek, L. K. *Nature* **313**, 313–316 (1985).
- Nestler, E. J. & Greengard, P. *Nature* **305**, 583–588 (1983).
- Nairn, A. C., Hemmings, Jr, H. C. & Greengard, P. *A. Rev. Biochem.* **54**, 931–976 (1985).
- Levitan, I. B. *J. Membrane Biol.* **87**, 177–190 (1985).
- Paupardin-Tritsch, D., Hammond, C. & Gerschenfeld, H. M. *J. Neurosci.* **6**, 2715–2723 (1986).
- Winquist, R. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7661–7664 (1984).
- Osterrieder, W. *et al. Nature* **298**, 576–578 (1982).

11. Brum, G., Flockerzi, V., Hofman, F., Osterrieder, W. & Trautwein, W. *Pflügers Arch. ges. Physiol.* **398**, 146-154 (1983).
12. Ewald, D. A., Williams, A. & Levitan, I. B. *Nature* **315**, 503-506 (1985).
13. Fesenko, E. E., Kolesnikov, S. S. & Lyubarsky, A. L. *Nature* **313**, 310-313 (1985).
14. Haynes, L. W. & Yau, K.-W. *Nature* **317**, 61-64 (1985).
15. Haynes, L. W., Kay, A. R. & Yau, K.-W. *Nature* **321**, 66-70 (1986).
16. Zimmerman, A. L. & Baylor, D. A. *Nature* **321**, 70-72 (1986).
17. Walter, Y., Miller, P., Wilson, F., Menkes, D. & Greengard, P. *J. biol. Chem.* **255**, 3757-3762 (1980).
18. Kostyuk, P. G. *Neuroscience* **5**, 945-959 (1980).
19. Hagiwara, S. & Byerly, L. A. *Rev. Neurosci.* **4**, 69-125 (1981).

Polymerase activity in lymphocyte culture supernatants from patients with Kawasaki disease

Jane C. Burns*, Raif S. Geha*,
Eveline E. Schneeberger†, Jane W. Newburger*,
Fred S. Rosen*, Laurie S. Glezen*, Alice S. Huang‡,
Joanne Natale† & Donald Y. M. Leung*§

* Divisions of Infectious Diseases, Allergy, Cardiology and Immunology, Children's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115, USA
† Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA
‡ Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

Kawasaki disease (KD) is an acute vasculitis of infancy and early childhood characterized by high fever, rash, mucositis, lymphadenopathy and coronary artery damage¹. Large epidemics have been described in Japan and the United States and the number of cases reported annually is steadily increasing^{2,3}. The aetiology of KD is unknown. During the acute phase of the disease marked immunologic alterations occur including generalized T-cell lymphocytopenia, activation of circulating T4⁺ helper T cells, decreased numbers of T8⁺ suppressor T cells and marked B-cell activation^{4,5}. We postulated that a lymphotropic virus with affinity for endothelial and lymphoid cells might explain the vasculitis and immunological abnormalities in KD. We report here our study of the particulate fraction from culture supernatants of peripheral blood mononuclear cells (PBMC) for evidence of retrovirus-associated reverse transcriptase (RT) activity. Activity was found in the supernatants from KD patients but not control cultures. This RT activity was transmitted to an established T-cell line (HUT-78) and thus may be due to an exogenous agent infecting KD lymphocytes.

A total of 17 cultures were established from the PBMC of 14 patients with KD. The cultures were initiated between 2 and 27 days after the onset of KD. The particulate fractions of culture supernatants from different times were assayed for RT polymerase activity. Culture supernatants from PBMC of KD patients showed a peak in polymerase activity between days 10 and 17 of culture (Fig. 1). In contrast, culture supernatants of PBMC from febrile controls contained no such activity. Polymerase activity in supernatants at day 10-13 of culture was significantly higher in the KD patients compared with controls ($P=0.02$ by the two-tailed rank sum test).

Figure 2 shows the peak polymerase activity measured for each culture. Maximal polymerase activity was significantly higher for KD patients than for controls (mean $\pm$ s.e.m. of dTMP incorporated was 15.3 ± 5.7 pM for KD and 3.2 ± 0.5 pM for controls; $P<0.05$ by two-tailed unpaired t -test). Culture supernatants of PBMC from patients with other diseases characterized by circulating activated T cells bearing HLA-DR⁺ antigens (systemic lupus erythematosus, severe atopic dermatitis and

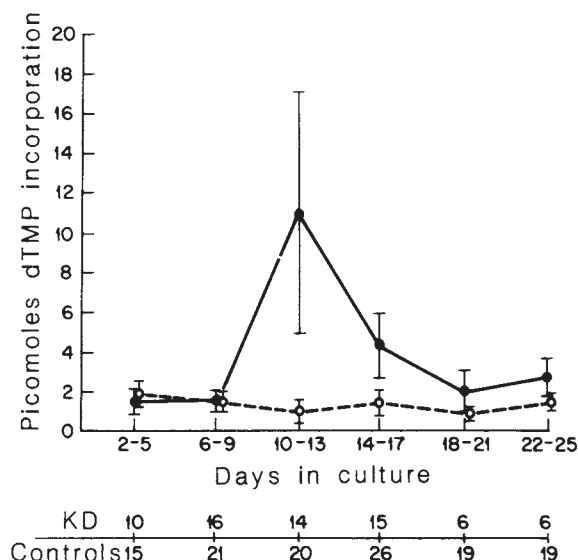


Fig. 1 Polymerase activity in PBMC culture supernatants. ●, Patients with KD; ○, controls. Below, number of cultures sampled at each time interval. PBMC were from patients diagnosed as having KD by J.B. or J.N. using standard clinical criteria^{1,2}. The mean age of the 14 patients studied was 35.7 months (range 19-61 months). Control patients (27 in all) were either hospitalized patients age 9 days-19 months with fever and with known bacterial (6), viral (2), or suspected viral (4) illnesses; patients aged 14 months to 30 yrs with non-infectious gastrointestinal, hepatobiliary, or allergic conditions (afebrile controls, 6) or patients with circulating DR⁺-T cells (marker for T cell activation) (3 adults with systemic lupus erythematosus, 5 patients with atopic dermatitis (aged 6 months to 9 yrs) and one patient (age 5 years) with acute onset of systemic juvenile rheumatoid arthritis). **Methods.** PBMC were separated from heparinized venous blood by Ficoll-Hypaque density-gradient centrifugation and cultured at 0.5×10^6 cells per ml supplemented with 2 mM glutamine and 10% fetal calf serum. Polybrene (1 μ g/ml), 10% (v/v) partially purified interleukin-2 (IL-2) (Collaborative Biotech, Cambridge, Massachusetts) with trace amounts of phytohemagglutinin (PHA) and antibody to α -interferon (Interferon Sciences, New Brunswick, New Jersey) at 200 U ml⁻¹ were added. Supernatants were collected at 3-4-day intervals and the cells were maintained at $0.5-2.0 \times 10^6$ cells per ml. Culture supernatants were centrifuged at 9,000g for 30 min, the supernatant was removed and re-centrifuged at 100,000g for 90 min (Beckman L8-55 ultracentrifuge; SW 41 rotor) and the resulting pellet was resuspended in 50 μ l buffer (Buffer A; 50 mM Tris/HCl pH 7.5, 20% (v/v) glycerol, 1 mM dithiothreitol (DTT), 250 mM KCl and 0.25% (v/v) Triton X-100), then snap frozen and thawed twice and 15 μ l aliquots stored at -70 °C before assay. Positive control supernatants included the HTLV-I cell lines MJ, HUT-102 and MT-2 kindly provided by Dr Myron Essex and Drs Flossie Wong-Staal and Robert Gallo. Reverse transcriptase was assayed by thawing 15 μ l specimens and incubating at 37 °C for 2 h with 40 μ l of the following reaction mixture: 0.125 μ Ci ³²P-dTTP (NEN) specific activity 600-800 Ci mmol⁻¹, 62.5 mM Tris/HCl, pH 8.0, 6.25 mM DTT, 0.625% Triton X-100, 10 mM dTTP (Boehringer Mannheim) (some assays were performed with all 4 dNTPs), 0.078 μ g μ l⁻¹ poly(rA):oligo(dT) (Sigma), and 7.5 mM MgCl₂. The reaction was stopped by addition of 10 mM disodium pyrophosphate and yeast RNA (1 mg ml⁻¹). Incorporation of ³²P-dTMP was measured by TCA precipitation. Filters were soaked in liquid scintillant (Biofluor, NEN) and counted in a Beckman model LS-1801 scintillation counter. Picomoles of dTMP incorporated were calculated from d.p.m. (specific activity: 400-850 cpm pmole⁻¹). Background (Buffer A alone) for each assay was subtracted. The mean of two assays on each specimen was used for statistical comparisons. The dTMP incorporation (mean $\pm$ s.e.m.) for the particulate fraction of the HTLV-I cell supernatants used as positive controls for each assay was 17.7 ± 1.8 pM (MJ), 24.4 ± 2.9 pM (HUT-102), and 18.2 ± 6.9 pM (MT-2).

juvenile rheumatoid arthritis) showed no increase in polymerase activity compared with controls ($P>0.1$).

The results of experiments to characterize the polymerase activity are given in Table 1. Heat treatment (80 °C for 10 min) of the pellet, omission of template and primer (poly rA:oligo dT) from the reaction mix, and omission of exogenous divalent cation in the presence of EDTA all greatly reduced the polymerase activity of both the positive HTLV-I control (MT-2) and the KD lymphocyte culture supernatants. Substitution of Mn²⁺ (0.5 mM) for Mg²⁺ reduced the polymerase

§ Address correspondence to D.Y.M.L. at: Division of Allergy, The Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115, USA.

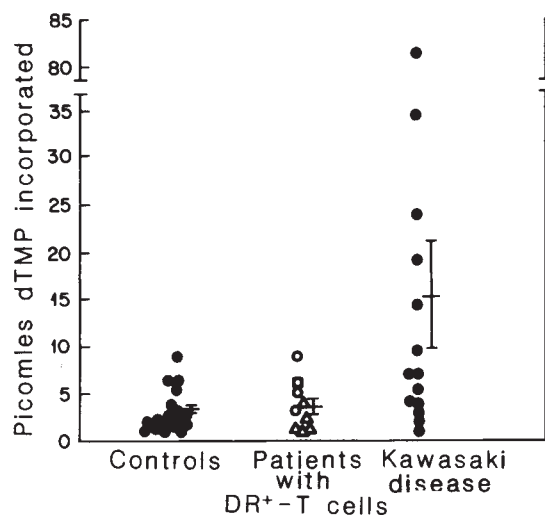


Fig. 2 Peak polymerase activity in the particulate fraction of PBMC culture supernatants. Patients with circulating DR⁺-T cells: ○, systemic lupus erythematosus ($n=3$); △, atopic dermatitis ($n=5$); and □, juvenile rheumatoid arthritis ($n=1$). Assays were performed as described in Fig. 1.

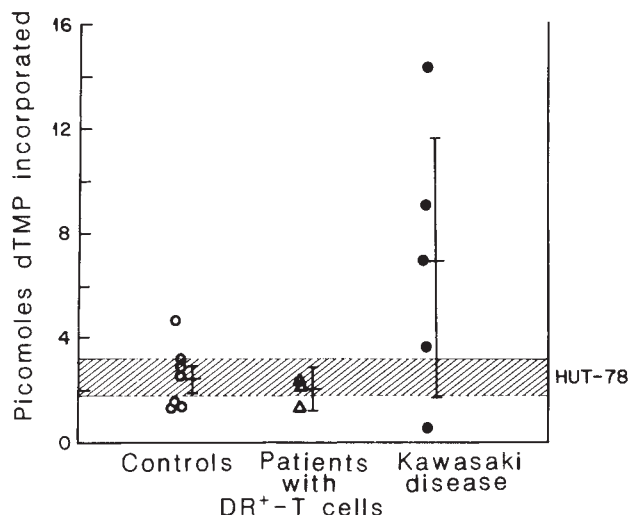


Fig. 3 Peak polymerase activity in lymphocyte/HUT-78 co-culture supernatants. Cultured PBMC from KD patients and controls were irradiated (10,000 rads) on culture day 3–15, and incubated at a density of 5×10^6 cells per ml with polybrene-treated, PHA-stimulated HUT-78 cells (density: $1-2 \times 10^6$ cells per ml). The cultures were maintained and collected in the same way as the PBMC cultures (Fig. 1). Control patients were 3 febrile patients with bacterial illness and 4 afebrile patients with asthma (○). The patients with DR⁺-T cells were 3 afebrile patients with atopic dermatitis (△). Cross-hatching, mean $\pm$ s.e.m. (2.5 ± 0.7 picomoles) for the supernatants of the HUT-78 cell line alone.

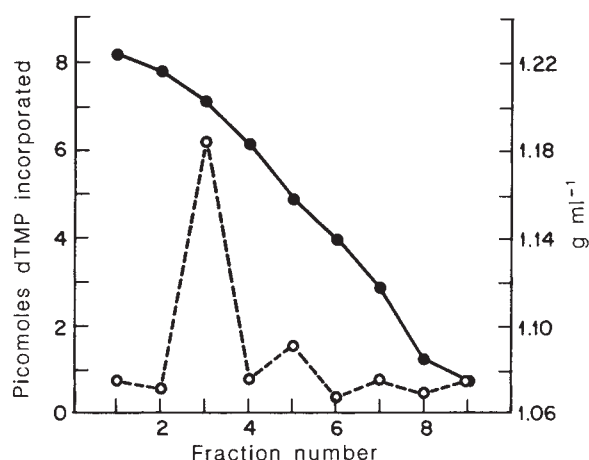


Fig. 4 Density of the particulate fraction associated with polymerase activity. ●, density; ○, picomoles of dTMP incorporated. Supernatants from 3 co-cultures of a single KD patient were pooled and concentrated by centrifugation (Beckman SW27 rotor; 25,000 r.p.m. for 90 min at 4°C). The pellet was resuspended in 100 μ l of a mixture containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl and 1 mM EDTA, pH 7.8 (TNE), layered onto a 20–60% sucrose gradient in TNE and centrifuged (Beckman SW 50.1 rotor; 35,000 r.p.m. for 16 h at 4°C). Fractions (500 μ l) were collected from the bottom, diluted in TNE, centrifuged (Beckman SW 50.1 rotor; 35,000 r.p.m. for 90 min at 4°C) and the pellet was resuspended in Buffer A and assayed for reverse transcriptase activity (Fig. 1 legend). Density of sucrose was determined by refractive index measurement.

Thus we have detected a polymerase activity that appears during the second week of culture in the supernatants of PBMC cultures from patients with KD. No such activity is associated with similar cultures from febrile patients or patients with other illnesses characterized by *in vivo* T-cell activation. Characteristics of the polymerase from KD cultures are consistent with a Mg^{2+} -dependent reverse transcriptase and suggest that a retrovirus may be the causative agent of KD. The profile of polymerase activity in the PBMC cultures over time is similar to that seen with human T-lymphotropic virus type I (HTLV-I) and human immunodeficiency virus (HIV)^{6,7}. The loss of polymerase activity may be explained by: (1) culture conditions that do not support continued growth of the agent; (2) depletion of a suitable host cell population through lytic infection; or (3) development of non-productive infection (latency) with no further production of infectious particles and their associated polymerase. Although our assays used conditions of pH, divalent cation concentration and a template/primer pair designed for an RNA-dependent DNA polymerase, it is possible that a polymerase that prefers a DNA template *in vivo* could use dTTP under these conditions. We have insufficient data to be sure of the template/primer specificity of this polymerase.

A lymphotropic virus could explain the transient alteration in lymphocyte populations observed during the acute phase of KD. The vasculitis could result directly from infection of the endothelial cells with the putative virus or by an indirect immune mechanism (for example, polyclonal B-cell activation with production of anti-endothelial cell antibodies⁸). Ample precedent for immune modulation exists among viruses known to be pathogenic in humans. Lymphotropic viruses such as Epstein-Barr virus (EBV), HTLV-I, HIV and cytomegalovirus (CMV) can alter the balance between helper and suppressor T cells, infect epithelial cell targets (nasopharyngeal cells in the case of EBV or vascular endothelium in the case of HTLV-I (ref. 9) and cause B-cell activation (EBV, HIV (ref. 10), CMV).

To date, we have examined PBMC from three patients with acute KD by transmission electron microscopy. Rare PBMC from all three of these patients contain viral particles in intracel-

activity by 60% (data not shown). We also investigated whether PBMC from KD patients could transmit this activity to the HUT-78 T cell line (Fig. 3). Three of the 5 co-cultures with KD PBMC showed significant polymerase activity ($>$ mean $\pm 2 \times$ s.e.m. of activity in culture supernatants from untreated HUT-78 cells) 7–12 days after co-cultivation. No activity peak was seen in 10 co-cultivation cultures of HUT-78 with PBMC from control patients. The supernatants from a KD PBMC co-culture were pooled and the density of the particles associated with the polymerase activity was determined by sucrose density gradient (Fig. 4). A single peak in activity was found at a buoyant density between 1.18 and 1.22 $g\ ml^{-1}$ of sucrose.

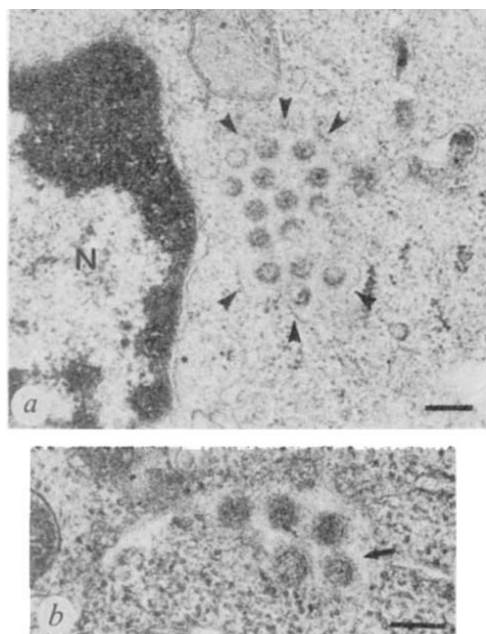


Fig. 5 Viral particles in cultured KD PBMC. *a*, Mononuclear cell containing; arrowheads, intracellular vacuole with many uniformly sized viral particles (105–118 nm), N, nucleus, (magnification $\times 47,500$, enlargement $\times 2.5$) scale bar = 200 nm. *b*, Intracellular vacuole at higher magnification ($\times 66,500$, enlargement $\times 3.5$). Arrow, viral particle budding from the vacuole membrane. Scale bar = 200 nm. KD PBMC were cultured for 7 days in IL-2-containing medium (Fig. 1 legend). Cell pellets were fixed in formaldehyde-glutaraldehyde for one hour, washed and post-fixed in 1.3% osmium tetroxide in 0.1 M *s*-Collidine buffer, pH 7.3. The cells were stained with 3% uranyl acetate in 75% ethanol and embedded in epon. Thin sections were then stained with lead citrate and examined in a Philips 301 electron microscope.

lular vacuoles (Fig. 5*a*). The particles are 105–118 nm in diameter, have an electron-dense core and an outer membrane and some appear to bud from the vacuole membrane (Fig. 5*b*). Similar viral particles were not observed in cultured PBMC from 5 control subjects. These preliminary observations support the possibility that the polymerase activity found in PBMC cultures of KD patients is associated with a virus.

Although the human retroviral illnesses caused by HTLV-I, -II and HIV are usually thought of as chronic syndromes, acute infection with HIV has been reported to be associated with a self-limited illness characterized by fever, rash, lympho-

dehopathy and mucositis¹¹. In contrast with most HTLV patients, the immune systems of KD patients return to normal after the acute illness is over. Further studies are needed to elucidate the possible importance of lymphocytotropic viruses in the pathogenesis of Kawasaki disease.

We thank Drs Ken McIntosh, David Hendricks and Jean Patterson for helpful discussion; Dr Susan Hoch, Dr Norbert Reidl, Nancy Wood, Maureen Maher, Julie Newton and Mary Fran McLane for technical advice and support; and David Lence and Barbara Connolly for manuscript preparation. This work was supported by National Research Service Awards AI-07202 and CA-09382, PHS grants HL 36781, AI-22058, AI-00440, AI-203732, and the National Foundation of the March of Dimes. R.S.G. is supported by Allergic Diseases Academic Award K07 AI-00440.

Received 18 April, accepted 9 September 1986.

1. Kawasaki, T. *Jap. J. Allerg.* **16**, 178–222 (1967).
2. Melish, M. E., Hicks, R. M. & Larson, E. J. *Am. J. dis. Child.* **130**, 599 (1976).
3. Bell, D. M. *et al. New Engl. J. Med.* **304**, 1568 (1981).
4. Leung, D. Y. M. *et al. Clin. Immun. Immunopath.* **23**, 100–112 (1982).
5. Leung, D. Y. M. *et al. J. Immun.* **130**, 2002 (1983).
6. Yoshida, M., Miyoshi, I. & Hinuma, Y. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2031–2035 (1982).
7. Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A. & Shimbabukano, J. M. *Science* **225**, 840–842 (1984).
8. Leung, D. Y. M. *et al. J. clin. Invest.* **77**, 1428–1435 (1986).
9. Hoxie, J. A., Mathews, D. M. & Cines, D. B. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7591–7595 (1984).
10. Popovic, M. *et al. Science* **226**, 459–462 (1984).
11. Cooper, D. A. *et al. Lancet* **i**, 537–539 (1985).

Antiviral effects of recombinant tumour necrosis factor *in vitro*

J. Mestan*, W. Digel†, S. Mitnacht*, H. Hillen‡, D. Blohm‡, A. Möller‡, H. Jacobsen* & H. Kirchner*

* Institute for Virus Research, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg 1, FRG

† Department of Internal Medicine III, University of Ulm, Steinhofstrasse 9, 7900 Ulm, FRG

‡ BASF AG, 6700 Ludwigshafen, FRG

Tumour necrosis factor (TNF) was first described¹ as a factor in the serum of mice injected with tubercle bacilli (BCG) and several days later with lipopolysaccharide (LPS). The gene encoding TNF has recently been cloned and pure recombinant human TNF is now available^{2,3}. TNF is known for its *in vivo* antitumour effect and *in vitro* cytotoxicity on certain transformed cell lines^{4,5}. Similarities in amino acid sequence and biological activity to lymphotoxin and cachectin have been reported^{2,6}, and very recently a growth-factor-like activity on diploid fibroblasts was observed⁷. There is no similarity between these proteins and interferons (IFNs), which are also induced during *in vivo* induction of TNF⁸. Here we describe the antiviral activity of pure recombinant human TNF in a typical *in vitro* antiviral assay which we discovered while investigating the possible role of TNF as an inducer of IFN.

Human laryngeal carcinoma cells (HEp-2) were grown in 96-well plates for 48 h. When cells had reached confluency, the medium was decanted and vesicular stomatitis virus (VSV) added at a multiplicity of infection (MOI) of 0.04. After 32 h, plates were fixed and stained. In the absence of TNF, we observed complete destruction of the cell monolayer, but pre-treatment with TNF leads to partial or complete protection. Concentrations as low as 1 ng ml⁻¹ inhibit the development of the virus-induced cytopathic effect (Table 1). As a control, we used IFNs as the effects of TNF are indistinguishable from those of IFNs. Using MOIs of VSV > 1, preincubation with TNF for at least 4 h was necessary to achieve protection. At a lower MOI of 0.04 it was even possible to partially protect the cells

Table 1 Characterization of polymerase activity

Patient lymphocyte culture		Picomoles of dTMP incorporated			-Mg ²⁺ + EDTA‡
		Complete reaction	Heat inactivation* & primer†	-Template	
C.N.	10 days	97.8	0	0.6	—
J.J./HUT-78	12 days	7.4	—	—	0.1
A.B./HUT-78	7 days	9.8	0	—	0.1
C.N./HUT-78	11 days	7.1	—	0.3	—
R.G./HUT-78	16 days	16.0	—	5.5	—
HUT/78		1.5	0.6	0.9	1.2
Control		0.3	0	0.4	0
Control/HUT-78		1.9	1.1	0.2	0.4
6 days					
MT-2 (HTLV-I cell line)		55.4	0	0.7	0
Buffer A		1.9	0.2	0.3	0.5

See Fig. 1 legend for reaction conditions.

* Heat inactivation of pellet was for 10 min at 80 °C before addition of reaction mixture.

† Template & primer (poly rA:oligo dT) omitted.

‡ MgCl₂ omitted, 1 mM EDTA (final concentration) added.

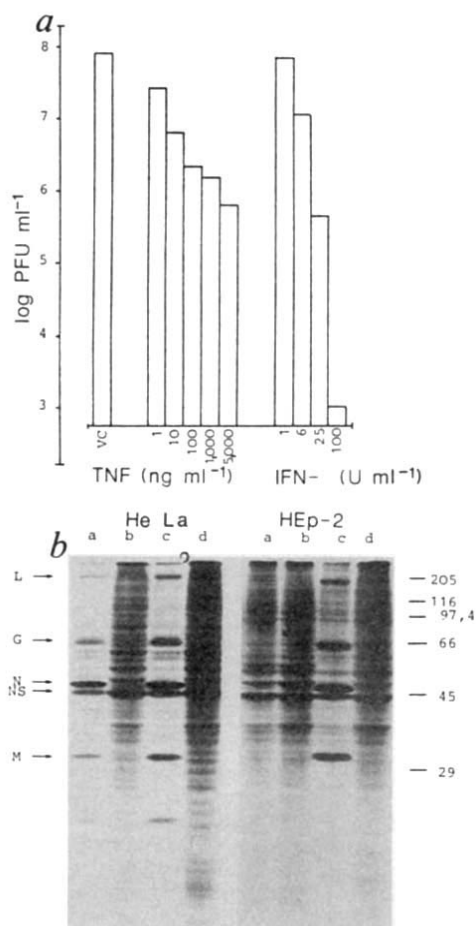


Fig. 1 Effect of TNF on virus yield (a) and viral protein synthesis (b). a, Virus yield reduction assay. HEP-2 cells, grown to confluency in microtitre plates, were overlaid with medium (MEM, 10% calf serum, CS) containing different doses of TNF or IFN for 20 h. Subsequently, cells were washed and infected with VSV in modified Eagle's medium (MEM) at a MOI of 0.04. After 1 h, the cell monolayers were washed twice and 200 μ l fresh MEM, 5% CS per well was added. (Incubation at 37 °C, 5% CO₂.) Cells were frozen 30 h post-infection at -70 °C. A plaque assay for determination of virus yields was performed by titration on confluent RITA-cell monolayers in 6-well plates. After freeze-thawing, supernatants containing virus were diluted with basal Eagle's medium (BEM) in 10-fold dilution steps and RITA-cell monolayers were infected with 200 μ l virus suspension per well. After infection (1 h) overlay medium (BEM, 1% carboxymethyl cellulose, 5% fetal calf serum) was added. Later (24 h) cells were fixed with 3% formaldehyde and stained with gentian violet and the plaques counted to determine plaque-forming units (PFU) ml⁻¹. b, Inhibition of the synthesis of viral proteins by TNF in HEP-2 or HeLa cells. Cells were grown to confluency in 24-well plates and incubated with either human IFN- β (500 U ml⁻¹) or TNF (5 μ g ml⁻¹) for 20 h followed by infection with VSV at a MOI of 10. After viral infection (4 h) medium was removed and replaced by methionine-free BEM containing ³⁵S-methionine at 35 μ Ci ml⁻¹ (150 μ l per well). The cells were pulse-labelled for 2 h, washed and dissolved in 100 μ l per well sample buffer for SDS-PAGE. Electrophoresis was performed in 12% gels according to Laemmli¹¹ (40 μ l of each sample). Gels were stained with Coomassie brilliant blue to visualize marker proteins and dried after treatment with enhancer (EN³HANCE, NEN). Autoradiographs of gels are shown. Lane a, TNF-treated, virus-infected cells; b, IFN-treated, virus-infected cells; c, virus control; d, cell control. Relative molecular mass (M_r) marker proteins ($\times 1,000$) SDS-6H high standard mixture (Sigma); VSV proteins: RNA-dependent RNA polymerase (L); glycoprotein (G); nucleoproteins (N, NS); nonglycosylated membrane protein (M).

Table 1 Cell protection assay showing the antiviral effect of TNF

TNF (ng ml ⁻¹)	HEL* Per cent of intact cell monolayer	Hep-2* Per cent of intact cell monolayer	Hep-2† Per cent of intact cell monolayer
1,000	100	100	ND
300	100	100	100
100	100	100	100
30	100	100	100
10	90	100	100
3	80	80	100
1	40	40	100
0.3	10	20	50
0.1	0	0	10

Cells were grown to confluency in microtitre plates. After treatment for 20 h with different concentrations of TNF batch I(*) or TNF batch II(†), overlaying medium was removed and VSV added at a MOI of 0.04 in 0.01 ml of serum-free medium. After infection (1 h) 0.1 ml complete medium was added. The protective effect of TNF was expressed as the extent of viable cell monolayers estimated after staining with gentian violet 32 h after infection and compared with an untreated, uninfected cell control (100%). The monolayers of untreated and infected cells (virus control) were completely destroyed (0%) under these conditions. TNF was produced by recombinant DNA methods and purified to homogeneity from bacteria as described previously^{2,3}. It was homogeneous by the criteria of SDS-polyacrylamide gel electrophoresis (PAGE)¹¹ and reverse-phase HPLC. Biological activity was measured by the L929 test⁵ and endotoxin content estimated by the LAL test¹². Two different batches of purified TNF were used: batch I, biological activity, 1×10^7 U mg⁻¹ protein; endotoxin content, >0.6 , <3.0 ng mg⁻¹ protein. Batch II, biological activity, 1.5×10^7 U mg⁻¹ protein; endotoxin content, >0.069 , <0.137 ng mg⁻¹ protein.

by adding TNF as late as 7 h after virus infection (data not shown). This suggests that TNF only protects cells that are not yet infected.

The antiviral effect of TNF is not limited to VSV but can be demonstrated with two additional viruses, encephalo-myocarditis virus (EMCV) and herpes simplex virus (HSV). TNF not only prevents the development of the cytopathic effect but in cells pretreated with different doses, virus yield is reduced in a dose-dependent manner (Fig. 1a). Like interferon, TNF intervenes at an early stage of viral replication as shown by the complete inhibition of the formation of viral proteins in HEP-2 cells pretreated with TNF or IFN (Fig. 1b). So far, we have demonstrated the antiviral effect of TNF with three out of six cell lines of human origin and with one out of four cell lines of animal origin: HEP-2, human embryonic lung fibroblasts (HEL), human embryonic lung fibroblasts (WI-38) and mouse embryonic fibroblasts (MEF) are susceptible to the antiviral effect of TNF, whereas human cervix carcinoma cells (HeLa), human skin fibroblasts (BUD-8) and human amnion cells (WISH) as well as mouse transformed fibroblasts (L929), rat embryonic fibroblasts (REF) and monkey kidney cells (RITA) are not protected against viral infection by pretreatment. Susceptibility to the antiviral activity was not restricted to cells with transformed phenotype. There is no indication that treatment with doses of TNF active in antiviral protection (up to 3 μ g ml⁻¹) have any negative effect on cell viability of uninfected Hep-2 cells. Neither cell growth nor plating efficiency is affected by the TNF doses used. However, when assayed for the susceptibility to the toxic effect of TNF in the presence of actinomycin D, HEP-2 cells show similar sensitivity to L929 cells (data not shown). Yet the latter are not protected against viral infection with VSV by pretreatment with TNF alone, suggesting that the antiviral and the cytotoxic effects of TNF are separate activities.

In experiments using antibodies capable of inhibiting the activities of human IFNs, we found that anti-IFN- α or anti-IFN- γ added to HEP-2 cells simultaneously with TNF does not affect the TNF-mediated antiviral protection. Anti-IFN- β results in a partial reduction of this effect. However, it is not possible to neutralize completely the antiviral effect of TNF even with high

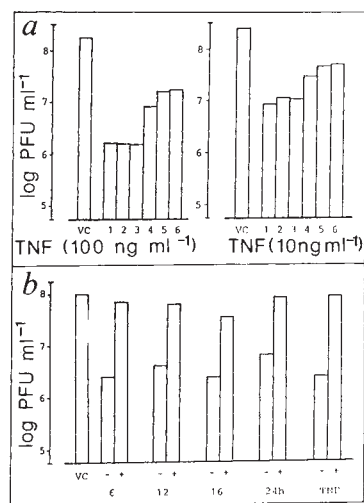


Fig. 2 Reversal of TNF-induced antiviral activity. *a*, Neutralization of the TNF antiviral effect by anti-human IFN antibodies; *b*, antiviral activities in the supernatants of TNF-treated Hep-2 cells.

Methods. *a*, TNF was mixed with anti-human IFN antibodies (previously confirmed to inhibit the antiviral activities of interferons) to final concentrations of 100 ng ml⁻¹ TNF or 10 ng ml⁻¹ TNF in MEM, 10% CS. Final concentrations of polyclonal antibodies (Paesel, Frankfurt) expressed in neutralizing units (NU) per ml were: (1) 0, TNF control; (2) 500 NU ml⁻¹, anti-IFN- α ; (3) 500 NU ml⁻¹, anti-IFN- γ ; (4) 50 NU ml⁻¹, anti-IFN- β ; (5) 500 NU ml⁻¹, anti-IFN- β ; and (6) 2,500 NU ml⁻¹, anti-IFN- β . Triplicates of each of these mixtures were added to confluent Hep-2 cells in microtitre plates and incubated at 37 °C (5% CO₂). Cells were then infected with VSV and virus yields were determined with the plaque assay described in Fig. 1*a*. VC, Virus control. *b*, Hep-2 cells grown to confluency in microtitre plates were incubated with 100 μ l per well MEM, 10% CS containing 200 ng ml⁻¹ TNF. After 6, 12, 16 and 24 h incubation, supernatants of 10 wells were collected. One aliquot of each supernatant (-) was mixed with an equal volume of medium (MEM, 10% CS), another aliquot (+) with an equal volume of medium containing 20 μ g ml⁻¹ monospecific anti-TNF antibody (BASF) to neutralize the activity of residual TNF. (10 μ g ml⁻¹ anti-TNF antibody completely neutralizes the antiviral activity of 100 ng ml⁻¹ TNF as shown in the two right-hand columns.) After 2 h at room temperature these mixtures were added to fresh Hep-2 cells in microtitre plates (in triplicate) and incubated for 20 h (37 °C, 5% CO₂). Infection with VSV and determination of virus yields was performed as described in Fig. 1*a*.

doses of anti-IFN- β (Fig. 2*a*). Most of the antiviral activity of supernatants from TNF-treated Hep-2 cells can be neutralized by anti-TNF antibodies (Fig. 2*b*), suggesting that there is only very little IFN present in the supernatants. The maximal remaining antiviral activity observed led to 0.5 log virus yield reduction, comparable to the effect of 2–3 U ml⁻¹ of external IFN- β added to Hep-2 cells before infection with VSV.

We next demonstrated that the interferon-induced enzyme (2'–5')(A)_n synthetase can be induced in Hep-2 cells grown to full confluency in a dose-dependent manner by treatment with doses active in antiviral protection. In cells 80–90% confluent the (2'–5')(A)_n synthetase level is not enhanced by treatment with TNF (Fig. 3), in agreement with an experiment where TNF-induced antiviral activity is compared in Hep-2 cells at different states of confluency and showing that fully confluent cells are most susceptible to the antiviral effect of TNF (data not shown). Induction of this enzyme can be regarded as an indication of the presence of interferon. However, using Northern blot techniques and specific probes for human IFN- α and IFN- β genes, we failed to detect any IFN-specific RNA in Hep-2 cells treated with various doses of TNF (data not shown).

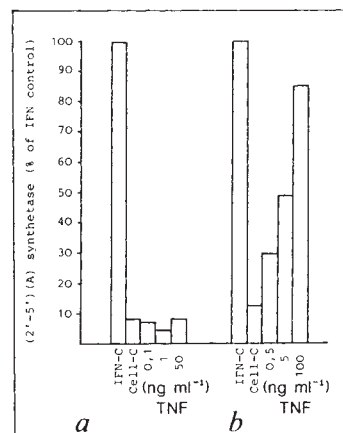


Fig. 3 Assay for IFN-induced (2'–5')(A)_n synthetase in Hep-2 cells. Assay for induction of (2'–5')(A)_n synthetase by TNF and IFN was performed as described elsewhere¹³. Briefly, cells were grown in 14-cm Petri dishes to ~80–90% confluency (*a*) or to full confluency (*b*) and incubated with human IFN (300 U ml⁻¹) or different concentrations of TNF (batch I) for 20 h. After collecting and washing, the cells were lysed in Nonidet P-40-containing lysis buffer and centrifuged at 12,000g for 10 min at 4 °C. Protein content of the supernatants was determined with the Bio-Rad protein assay kit¹⁴ with bovine serum albumin as protein standard. Protein (200 μ g) from each supernatant was added to 20 μ l poly(IC)-agarose (P.L. Biochemicals) followed by incubation for 1 h at room temperature and three washes in lysis buffer to remove unbound material. Assay mix (15 μ l) containing 5 mM ATP (25 μ Ci [α -³²P]ATP per μ mol ATP) was added and incubated for 6 h at 30 °C. Subsequently the poly(IC)-agarose was removed by centrifugation and 15 μ l supernatant treated with alkaline phosphatase. Inorganic phosphate was adsorbed on alumina by passing the samples through small alumina columns under acidic conditions (pH 2). The amount of oligo (2'–5')(A)_n (³²P-labelled) was evaluated by determining the c.p.m. in the eluates in a liquid scintillation counter.

After submitting our manuscript we became aware of data of the group of Jan Vilcek showing the induction of IFN- β by TNF in serum-starved human foreskin fibroblasts leading to inhibition of EMCV replication in these cells. As anti-human IFN- β antibodies can neutralize this effect they concluded that IFN- β is the mediator of the antiviral activity of TNF⁹.

Our data (Fig. 2) only partially agree with this conclusion. Although TNF seems to be a weak inducer of IFN- β -like activity, the amount of the detectable IFN in the supernatants of TNF-treated Hep-2 cells is not sufficient to account for the observed virus yield reductions in these cells. Therefore, we propose that the antiviral effect of TNF is not exclusively mediated by the induction of IFN but that there is an additional mechanism caused by the action of TNF itself. Both TNF and the TNF-induced interferon may act together in establishing the antiviral state of the cells.

The mechanisms of TNF-induced antiviral protection need to be explored further to determine whether they are similar to the known mechanisms of antiviral activity of interferon. Note that TNF appears to be the only lymphokine apart from interferon that protects cells against viral infection. Old¹⁰ recently suggested that the physiological role of TNF is that of a protective agent against infectious diseases and this view is supported by our present findings.

Received 28 January; accepted 20 August 1986.

1. Carswell, E. A. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **72**, 3666–3670 (1975).
2. Pennica, D. *et al.* *Nature* **312**, 722–729 (1984).
3. Aggarwal, B. B. *et al.* *J. biol. Chem.* **260**, 2345–2354 (1985).
4. Helson, L., Green, S., Carswell, E. & Old, L. J. *Nature* **258**, 731–732 (1985).
5. Ruff, M. R. & Gifford, G. E. *Infect. Immun.* **31**, 380–385 (1981).
6. Beutler, B., Milsark, I. W. & Cerami, A. C. *Science* **229**, 869–871 (1985).

7. Vilcek, J. *et al.* *J. exp. Med.* **162**, 632-643 (1986).
8. Younger, J. S. & Stinebring, W. R. *Nature* **208**, 456-458 (1965).
9. Kohase, M., Henriksen-De Stefano, D., May, L. T., Vilcek, J. & Sehgal, P. B. *Cell* **45**, 659-666 (1986).
10. Old, L. J. *Science* **230**, 630-632 (1985).
11. Laemmli, U. K. *Nature* **227**, 680-685 (1970).
12. *Fed. Reg.* 4.11.1977, 42FR57749, FDA.
13. Merlino, G., Revel, M. & Wallach, D. *Analyt. Biochem.* **110**, 190-196 (1980).
14. Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).

Tumour necrosis factors α and β inhibit virus replication and synergize with interferons

Grace H. W. Wong & David V. Goeddel

Department of Molecular Biology, Genentech, Inc.,
460 Point San Bruno Boulevard, South San Francisco,
California 94080, USA

Tumour necrosis factor (TNF) and lymphotoxin were initially described as tumoricidal proteins that are produced by activated macrophages^{1,2} and lymphocytes^{3,4}, respectively. Since TNF and lymphotoxin are structurally related, bind to the same cell surface receptor⁵ and have indistinguishable biological activities^{6,7}, they have been designated as TNF- α and TNF- β , respectively⁸. The multiple activities⁸⁻¹⁵ of these molecules indicate their importance in immunoregulatory responses. Here we report that both TNF- α and TNF- β have antiviral activity and synergize with interferons (IFNs) in the induction of resistance to both RNA and DNA virus infection in diverse cell types. These effects of TNFs are not due to the induction of IFN synthesis. Virus-infected cells are selectively killed by TNFs and this activity is accelerated by IFN- γ . The production of TNFs is induced by viruses, further suggesting the importance of TNFs in the physiological antiviral response.

During an investigation of the effects of human TNFs on the production of IFN- γ by human lymphocytes, we observed that purified recombinant TNF- α and TNF- β had IFN-like activity and inhibited virus-mediated cytopathic effects. Pretreatment of a human renal carcinoma cell line (7860) with TNF- α caused a dramatic dose-dependent inhibition in viral replication, as

determined by measuring virus yield after infection. These results were observed with both RNA viruses (encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV)) and DNA viruses (adenovirus-2 (Ad-2) and herpes simplex virus type II (HSV-2)) (Fig. 1). Similar results were found with TNF- β and no qualitative or quantitative difference between the two TNF types was observed (Fig. 1). Interestingly, the relative antiviral potencies of TNFs and IFN- γ varied with different viruses. TNF- α or TNF- β alone was able to inhibit VSV replication, whereas IFN- γ alone had no effect (Fig. 1a). TNFs were less potent (20-40-fold) than IFN- γ in protecting against EMCV (Fig. 1b) but were more potent (10-20-fold) in protecting against Ad-2 and HSV-2 (Fig. 1c, d). The antiviral activities of TNF- α and TNF- β were neutralized by the appropriate specific monoclonal antibodies but not by neutralizing antibodies specific for IFN- α , - β or - γ (Fig. 1a).

Antiviral effects of TNFs were observed with some but not all cell types. TNFs were also effective against VSV replication in the human cell lines RPMI-8226 (myeloma), and U87MG (glioblastoma), C127 mouse epithelial cells and Rat-1 fibroblasts. These results show that TNF- α and TNF- β have intrinsic

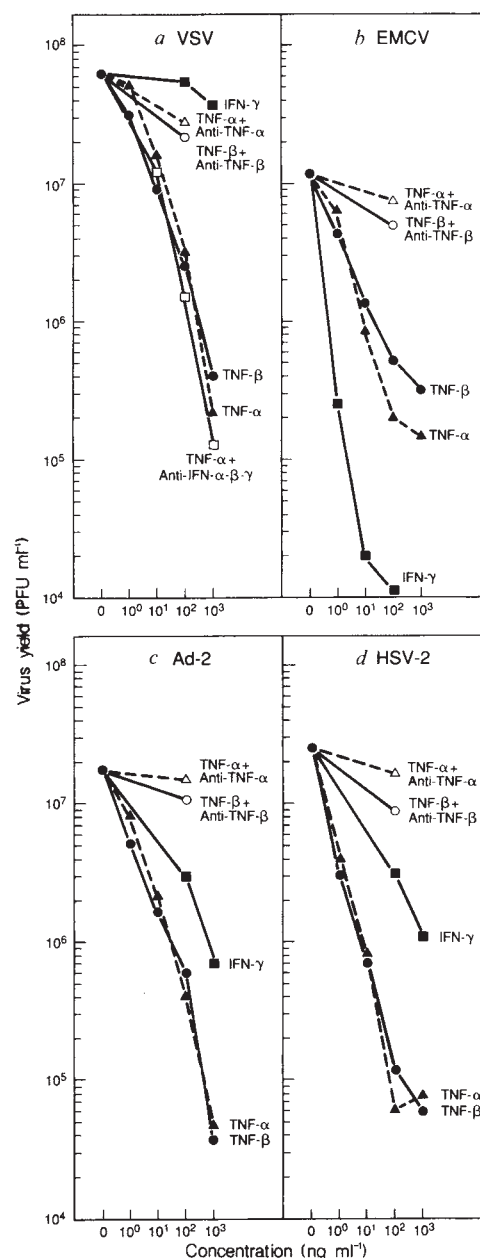


Fig. 1 TNF- α and TNF- β inhibit virus yield in 7860 cells. Human 7860 renal carcinoma cells (5×10^5 cells ml^{-1}) were seeded into 24-well tissue culture plates, treated with purified recombinant human TNF- α or TNF- β , at indicated concentrations or purified recombinant human IFN- γ in the presence or absence of antibodies. After 24 h incubation, the supernatants were removed before inoculation with VSV, EMCV, Ad-2 or HSV-2 viruses at multiplicities of infection (MOIs) of 10, 1, 100, and 100, respectively. After 2 h, the supernatants were aspirated to remove unadsorbed virus and the cells were incubated at 37°C in medium containing 5% fetal bovine serum (FBS). After 24 h (VSV and EMCV) or 48 h (Ad-2 and HSV-2), the cultures were frozen and thawed twice to lyse the cells, centrifuged at 400g for 10 min and assayed for infectious virus yield in terms of plaque-forming units per ml (PFU ml^{-1})²⁷ on A549 cells. EMCV, Ad-2 and HSV-2 were propagated in A549 cells, while VSV was grown in BHK cells, and the viral titres were determined as described²⁷. The VSV and EMCV virus stocks were purchased from the American Type Culture Collection. Ad-2 and HSV-2 were obtained from Dr K. Anderson (Genentech, Inc.). TNF- α or TNF- β at $1 \mu\text{g ml}^{-1}$ had undetectable toxic effects on uninfected 7860 cells. For neutralization experiments, $0.1 \mu\text{g ml}^{-1}$ of TNF- α or TNF- β was co-incubated with the corresponding anti-TNF and a mixture of anti-IFN- α , - β , - γ antibodies, respectively, for 4 h at 37°C before adding to the cells. Polyclonal antibodies to IFN- α and - β were purchased from Boehringer and Lee Biomolecular Research Inc., whereas purified monoclonal antibodies against TNF- α , TNF- β and IFN- γ were generated at Genentech, Inc. The concentration of each anti-TNF monoclonal antibody used was 10-fold higher than that required to completely neutralize TNF cytotoxic activity. Similarly, the concentration of anti-IFN antibodies used was sufficient to neutralize a 10-fold excess of the homologous IFNs. The specific activities for purified recombinant human TNF- α and TNF- β were 3×10^7 - 4×10^7 units mg^{-1} determined in a standard cytotoxic assay using LM cells²⁸. The specific activities for purified recombinant human IFN- α , IFN- β and IFN- γ were 2×10^8 units mg^{-1} , 3×10^7 units mg^{-1} and 4×10^7 units mg^{-1} , respectively, determined in a standard cytopathic effect inhibition assay using human A549 cells challenged with EMCV.

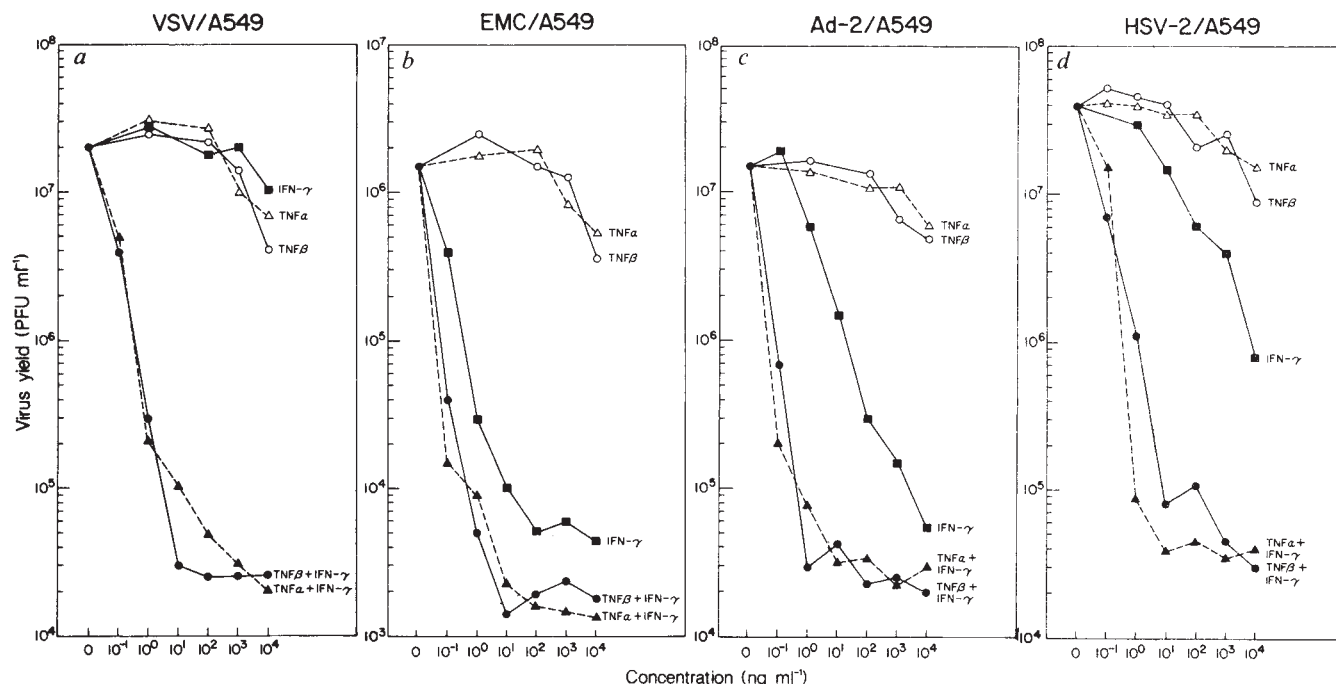


Fig. 2 TNFs enhance the ability of IFN- γ to inhibit virus replication. Confluent A549 cells were treated with the TNF- α , TNF- β , IFN- γ or the indicated concentrations for 18 h before challenge with VSV (a), EMCV (b), Ad-2 (c) or HSV-2 (d) at MOIs of 10, 1, 100 and 100, respectively. The inhibition of virus yield was determined as described in Fig. 1.

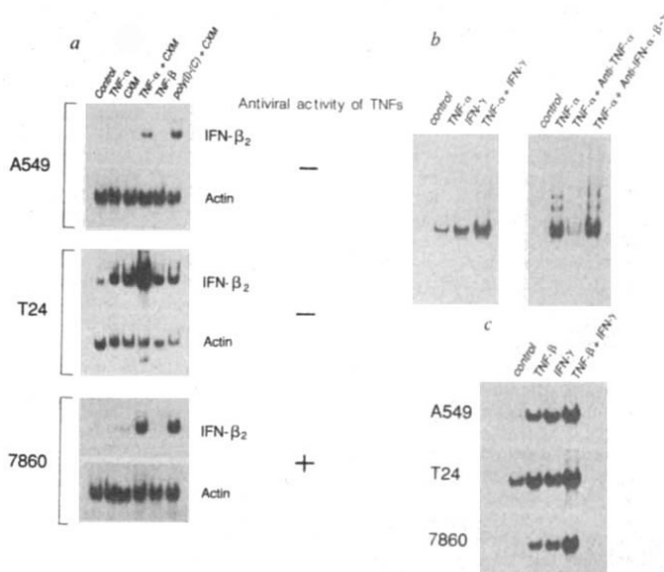
antiviral activity and can induce an IFN-like cellular state of viral resistance.

Since IFNs have been shown to enhance the cytotoxic activity of TNFs^{16,17}, we examined the effect of TNFs on the antiviral activity of IFNs. IFNs protect against VSV infection in most cells¹⁸, but purified recombinant human IFN- γ has little anti-VSV activity when assayed on the A549 human lung carcinoma cell line. However, in the presence of purified recombinant human TNF- α or TNF- β , IFN- γ was able to protect A549 cells from VSV-mediated cytopathic effects (data not shown). Unlike IFN- γ , IFN- α and IFN- β alone have some anti-VSV activity on A549 cells. This activity was also enhanced by TNF- α , although the total anti-VSV activity was no greater than that seen with TNF- α and IFN- γ (data not shown). Thus, the antiviral enhancing activity of TNFs is not restricted to IFN- γ .

The antiviral enhancing activity of TNFs can also be observed by measuring reduction in virus yield. Neither TNF nor IFN- γ alone inhibited VSV replication in A549 cells (Fig. 2a). However, TNF in combination with IFN- γ caused a large reduction in virus yield (about 2,000-fold greater than observed for IFN- γ alone). A 99% reduction required only 1 ng ml⁻¹ of each cytokine in combination. Furthermore, TNF- α and TNF- β were able to enhance the activity of IFN- γ against EMCV in A549 cells, especially at low concentrations of IFN- γ (Fig. 2b).

The synergistic antiviral effect of TNF- α or TNF- β is not restricted to RNA viruses but is also observed with DNA viruses such as Ad-2, Ad-5, HSV-1 and HSV-2. The combination of TNF- α or TNF- β with IFN- γ strongly inhibited replication of Ad-2 and HSV-2 in A549 cells (Fig. 2c and d). At concentrations higher than those required for protection against EMCV, IFN- γ

Fig. 3 Regulation of 'IFN- β_2 ' mRNA and induction of 2-5A synthetase mRNA by TNFs. a, A549, T24 and 7860 cells were incubated with TNF- α (0.1 μ g ml⁻¹), TNF- β (0.1 μ g ml⁻¹), cycloheximide (CXM) (1 μ g ml⁻¹), poly(I)·poly(C) (10 μ g ml⁻¹), or the indicated combinations. Total cytoplasmic RNA was extracted²⁹ after 4 h exposure to TNFs. Poly(A) RNA was prepared by oligo(dT) cellulose³⁰. Electrophoresis and Northern hybridization using 0.7 μ g RNA per lane, were performed as described³¹. ³²P-labelled 'IFN- β_2 ' was used as hybridization probe. The probe was prepared using DNA polymerase I (Klenow fragment) to fill in the 3' ends of overlapping synthetic 45- and 42-mers and corresponds to nucleotides 32 to 55 of the 'IFN- β_2 ' sequence³². As a control, the same RNA filter was also hybridized with ³²P-labelled α -actin (ref. 33) cDNA probe to indicate that the levels of mRNA on each lane were similar. b, TNF- α induces the expression of 2-5A synthetase mRNA. Confluent A549 cells treated with TNF- α (0.1 μ g ml⁻¹), IFN- γ (0.1 μ g ml⁻¹) or the combination; treated with TNF (0.1 μ g ml⁻¹) with or without excess monoclonal anti-TNF- α or a mixture of a 10-fold excess polyclonal antibodies against IFN- α , - β or - γ . c, Confluent A549, T24 and 7860 cells treated with TNF- β (0.1 μ g ml⁻¹) or IFN- γ (0.1 μ g ml⁻¹) or the combination. Total cytoplasmic RNA was extracted. Poly(A) RNA was prepared and Northern hybridization was performed after 12 h treatment. Poly(A) RNA (~1 μ g per lane) was hybridized with ³²P-labelled 2-5A synthetase probe³⁴, which was prepared by filling in at the 3' end of two overlapping synthetic 45- and 42-mers corresponding to nucleotides 331 to 395 of the 2-5A synthetase cDNA sequence³⁴.



alone provided partial protection against Ad-2 and HSV-2, whereas TNF- α or - β alone did not. In the presence of low concentrations of IFN- γ (1 ng ml^{-1}), TNF concentrations as low as 1 ng ml^{-1} were sufficient to inhibit Ad-2 and HSV-2 virus yield (Fig. 2c and d). Thus, TNFs and IFN- γ together are more efficient in inhibiting both RNA and DNA virus yield than either molecule alone.

Various cell lines were tested for their ability to respond to the antiviral potentiating activity of TNFs. The antiviral enhancing activity of human TNFs was also observed on human (HT-1080, lung fibrosarcoma; HeLa cervical carcinoma; T24 bladder carcinoma; HT-29 colon carcinoma; 7860 renal carcinoma; ST-486 Burkitt's lymphoma; RPMI-8226 myeloma and U87MG glioblastoma) and non-human (MDBK bovine kidney fibroblast; Rat-1 rat fibroblast; C127 mouse epithelial cells and Raw-264 mouse macrophage) cell lines. The combination of IFN- γ and TNF- α resulted in inhibition of VSV replication from 50 to 4,000 times greater than IFN- γ alone in these cells. Thus, the antiviral potentiating activity of TNFs, as with their tumoricidal activity, appears to be species-nonspecific.

Three different experimental approaches indicated that the antiviral activities of TNF are not caused by the induction of IFNs. First, concentrated medium from cells pretreated with TNF contained no detectable antiviral activity. Second, antibodies specific for IFN- α , - β and - γ could not neutralize the anti-VSV activity of TNF- α in 7860 cells (Fig. 1a). Third, Northern hybridization of RNA from TNF-treated A549 cells with specific IFN- α ¹⁹, IFN- β ²⁰ and IFN- γ ²¹ complementary DNA (cDNA) probes failed to detect any of these mRNAs.

Recently, it was suggested that the action of TNF- α may be mediated through the induction of another molecule that has been termed 'IFN- β_2 '. The mRNA for 'IFN- β_2 ' can be induced by cycloheximide (CXM)²²⁻²⁴, but whether or not the 'IFN- β_2 ' translation product has antiviral activity remains controversial²⁵. Pretreatment of T24 cells with CXM for 2 h induced high levels of mRNA for 'IFN- β_2 ' (Fig. 3a) but resulted in no detectable antiviral protection. TNFs induced 'IFN- β_2 ' mRNA in T24 but not in 7860 or A549 cells (Fig. 3a), although they have antiviral activity in 7860 cells but not in T24 or A549 cells. The induction of 'IFN- β_2 ' mRNA was greater when cells were treated with both TNF- α and CXM than either alone (Fig. 3a). However, this treatment for 2 h followed by removal of TNF and CXM, and further incubation for 12 to 24 h, failed to protect against EMCV or VSV infection in T24 or A549 cells. These results show that there is no correlation between the antiviral activity of TNFs and the induction of 'IFN- β_2 ' mRNA. These experiments demonstrate that the antiviral activities of TNFs are direct and are not mediated through the induction of IFNs.

The synthesis of the enzyme 2',5'-oligoadenylate synthetase (2-5A synthetase) is strongly induced by all IFNs, making it a good marker of IFN activity²⁶. TNF- α , like IFNs, induces the expression of 2-5A synthetase mRNA in A549 cells (Fig. 3b). The mRNA is first detectable 4 h after induction by TNF- α , or IFN- γ , or the combination, and reaches its maximal level at 12 h. The induction of 2-5A synthetase mRNA by TNF- α was neutralized by monoclonal antibodies directed against TNF- α but not by antibodies against IFN- α , - β or - γ (Fig. 3b). This result further demonstrates that induction of 2-5A synthetase mRNA by TNF- α is not mediated through the production of IFNs. TNF- β can also induce 2-5A synthetase mRNA (Fig. 3c). The combination of TNFs and IFN- γ gave stronger induction than either cytokine alone (Fig. 3). Thus, TNFs share with IFNs the ability to induce 2-5A synthetase. However, induction of 2-5A synthetase alone is not sufficient to mediate antiviral protection in these cells because neither TNF nor IFN- γ alone protects against VSV in A549 cells, yet either alone induces significant levels of 2-5A synthetase mRNA.

In addition to enhancing the induction of an antiviral state in uninfected cells, TNFs are also able selectively to kill virus-infected cells. Under normal growth conditions in the absence

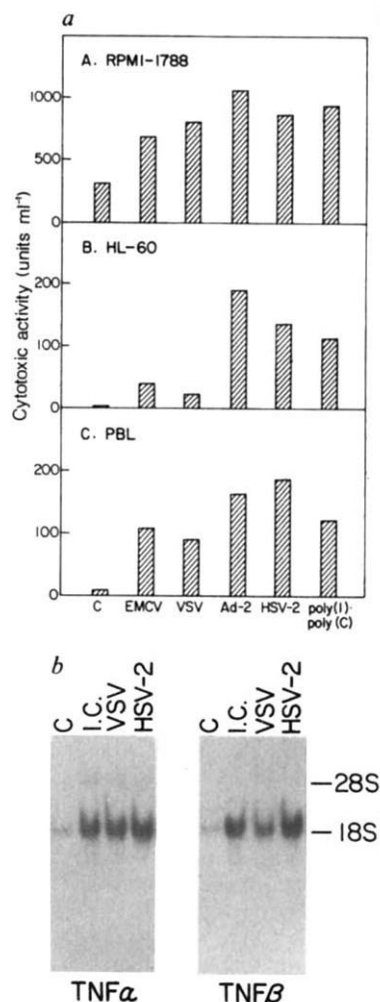


Fig. 4 Viruses and poly(I)·poly(C) induce TNF production. *a*, The human B lymphoblastoid TNF- β producing cell line RPMI-1788 (ref. 7) (A) and the TNF- α -producing cell line HL-60 (ref. 6) (B), and human peripheral blood leukocytes (C) isolated from Ficoll-hypaque gradients at a concentration of about 1×10^6 cells ml^{-1} were incubated with EMCV, VSV, Ad-2 or HSV-2, at MOIs of 1, 10, 100 and 100, respectively, or poly(I)·poly(C) ($10 \mu\text{g ml}^{-1}$) for 2 h, washed and further incubated in medium at 37°C . After 5 h, supernatants were collected and assayed for cytotoxic activity²⁸. *b*, Northern hybridization of RNA derived from human peripheral blood leukocytes (2×10^6 cells ml^{-1}) induced with poly(I)·poly(C) ($10 \mu\text{g ml}^{-1}$), VSV, or HSV-2 at MOIs of 10 and 100, respectively. RNAs extracted after 4 and 12 h infection were hybridized with ³²P-labelled TNF- α and TNF- β cDNA probes, respectively, which were ³²P-labelled *Ava*I-*Hind*III restriction fragment (578 bp) from human TNF cDNA⁶ and an *Eco*RI restriction fragment (~650 bp) from the coding region of human TNF- β cDNA⁷, respectively.

of virus infection, A549 cells are resistant to the cytotoxic activity of TNFs at 24 h even in the presence of IFN- γ . The viability of VSV-infected cells 12 and 18 h after virus infection was 92% and 70%, respectively, whereas the viability of TNF-treated, virus-infected cells had dropped to 30–40%. Similarly, TNFs selectively killed Ad-5-infected A549 cells (data not shown). Thus, virus-infected cells are more susceptible to lysis by TNFs than are uninfected cells, and lyse earlier than they would have in the absence of TNFs.

Although IFN- γ ($0.1 \mu\text{g ml}^{-1}$) alone did not selectively kill VSV-infected A549 cells, it accelerated the lysis of virus-infected cells by TNFs. Within 18 h, the combination of IFN- γ and TNFs had killed virtually all VSV-infected cells. An increase in vulnerability of virus-infected cells to killing by TNFs was also found with other cell lines, such as 7860 renal carcinoma and

U87MG glioblastoma cells. These results suggest that the selective killing by TNFs is a general phenomenon and not restricted to a specific cell type or virus.

Because TNFs potentiate the antiviral activity of IFNs, it was of interest to determine whether viruses could induce the production of TNFs. When the human B lymphoblastoid cell line RPMI-1788 (known to produce TNF- β ⁷) and promyelomonocytic leukaemia cell line HL-60 (known to produce TNF- α ⁶) were challenged with EMCV, VSV, Ad-2, or HSV-2, significant levels of TNFs were produced (Fig. 4a). Poly(I)·poly(C), an inducer of IFN- α and IFN- β synthesis, also stimulated the production of TNFs in these cells. Normal peripheral blood leukocytes also produced significant levels of TNF activity after exposure to these inducers (Fig. 4a). Northern hybridization of poly(A) RNA from leukocytes with TNF- α and TNF- β cDNA probes showed that mRNA for both these genes were induced by this treatment (Fig. 4b).

Our results show that TNF- α and TNF- β have intrinsic antiviral activity on certain cells and dramatically synergize with IFNs in terms of antiviral activities on diverse cell types. The mechanisms by which TNFs alone specifically induce an antiviral effect in certain cells is not known. However, cells (such as T24) that are not protected by TNFs do have TNF receptors^{5,9} and also respond to TNFs as measured by induction of 2-5A synthetase and 'IFN- β ' mRNA (Fig. 3).

The different spectra of antiviral activity of TNFs and IFN- γ , together with their synergistic actions, suggest that TNFs and IFN- γ exert their antiviral effects through at least partially independent mechanisms. The synergistic action of TNFs and IFN- γ is not due to induction of IFN- γ receptors because TNFs have no effect on the expression of IFN- γ receptors (Ramani Aiyer, personal communication). Further work is required to determine the mechanism of TNF-induced antiviral activity and its synergy with IFNs.

The data presented here suggest that a major function of TNFs may be to combat viral infection by at least two mechanisms: by inducing resistance in uninfected cells, and by selectively killing virus-infected cells in order to prevent viral spread. The production of TNFs and IFNs in response to viral infection, and their positive cooperation with each other, suggest that together they form a powerful and general host defence system. The potency and effectiveness of these molecules in combination holds promise for the future treatment of viral diseases.

We thank Drs I. Clark Lewis, C. W. Czarniecki, P. W. Gray and H. M. Shepard for valuable comments on this manuscript; the Process Development and Manufacturing group at Genentech for producing recombinant human TNFs and human and murine IFN- γ , C. Nelson for providing monoclonal antibodies against TNF- α and TNF- β , M. Bombara, S. Marsters and A. M. Jung for performing some of the bioassays, K. Monroe for virus stocks and protocols, A. Gray for artwork, and J. Arch for preparing the manuscript.

Received 10 June; accepted 6 August 1986.

1. Carswell, E. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3666-3670 (1975).
2. Pennica, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 6060-6064 (1985).
3. Ruddle, N. H. & Waxman, B. H. *Science* **157**, 1060-1062 (1967).
4. Granger, G. A. & Kolb, W. P. *J. Immunol.* **101**, 111-120 (1968).
5. Aggarwal, B. B., Essalu, T. & Hass, P. E. *Nature* **318**, 665-667 (1985).
6. Pennica, D. *et al. Nature* **312**, 724-729 (1984).
7. Gray, P. W. *et al. Nature* **312**, 721-724 (1984).
8. Shalaby, M. R. *et al. J. Immunol.* **135**, 2069-2073 (1985).
9. Sugarman, B. J. *et al. Science* **230**, 943-945 (1985).
10. Gamble, J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 8667-8671 (1985).
11. Silberstein, D. S. & David, J. R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1055-1059 (1986).
12. Beutler, B. *et al. Nature* **316**, 552-554 (1985).
13. Torti, F. M. *et al. Science* **229**, 867-869 (1985).
14. Bertolini, D. R. *et al. Nature* **319**, 516-518 (1986).
15. Dayer, J. M., Beutler, B. & Cerami, A. *J. exp. Med.* **162**, 2163-2168 (1985).
16. Williams, T. W. & Bellanti, J. A. *J. Immunol.* **130**, 518-520 (1983).
17. Lee, S. H. *et al. J. Immunol.* **133**, 1083-1086 (1984).
18. Stewart II, W. Z. *The Interferon System* (Springer, Vienna-New York, 1981).
19. Goeddel, D. V. *et al. Nature* **287**, 411-416 (1980).
20. Goeddel, D. V. *et al. Nucleic Acids Res.* **8**, 4057-4074 (1980).
21. Gray, P. W. *et al. Nature* **295**, 503-508 (1982).
22. Kohase, M. *et al. Cell* **45**, 659-666 (1986).

23. Weissenbach, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7152-7156 (1980).
24. Sehgal, P. B. & Sagar, A. D. *Nature* **288**, 95-97 (1980).
25. Content, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 2768-2772 (1982).
26. Wallach, D. *et al. Interferons, UCLA Symposia*, Vol. 25, 449-463 (Academic, New York, 1982).
27. Rager-Zisman, B. & Merigan, T. C. *Proc. Soc. exp. Med.* **142**, 1174-1179 (1973).
28. Kramer, S. M. & Carver, M. E. *J. immun. Meth.* (in the press).
29. Karin, M. *et al. Cell* **36**, 371-379 (1984).
30. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408-1412 (1972).
31. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
32. Haegeman, G. *et al. Eur. J. Biochem.* (in the press).
33. Minty, A. J. *et al. J. biol. Chem.* **256**, 1008-1014 (1981).
34. Benesh, P. *et al. EMBO J.* **4**, 2249-2256 (1985).

An activated Harvey *ras* oncogene produces benign tumours on mouse epidermal tissue

Dennis R. Roop*, Douglas R. Lowy†, Pierre E. Tambourin‡, James Strickland*, John R. Harper*, Michael Balaschak‡, Edwin F. Spangler‡ & Stuart H. Yuspa*

* Laboratory of Cellular Carcinogenesis and Tumor Promotion and

† Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892, USA

‡ Microbiological Associates, Bethesda, Maryland 20816, USA

Studies of the mutagenic action required for specific chemicals to produce benign or malignant tumours suggest that in mouse skin at least two genetic events occur before carcinoma formation¹. The isolation of an activated form of the *c-ras*^H gene from skin papillomas has provided evidence that this gene may be a target for the first mutation, which could constitute the initiating mutation in skin carcinogenesis^{2,3}. *In vitro* studies indicate that the *v-ras*^H gene of Harvey murine sarcoma virus (Ha-MSV), a replication-defective transforming retrovirus, could impart a conditional initiated phenotype on cultured keratinocytes by blocking their ability to differentiate terminally and arresting them in a late basal cell stage of maturation⁴. We now show that when the Ha-MSV *v-ras*^H gene is introduced into cultured keratinocytes by a defective retroviral vector, skin grafts constructed with cells carrying the mutated *ras* oncogene produce papillomas on athymic nude mouse recipients. Furthermore, the expression of the exogenous oncogene seems to be regulated at the transcriptional level in the differentiated portions of the benign tumour.

Mouse keratinocytes were cultured in medium containing 0.05 mM Ca²⁺, a condition which selects for basal cells⁵. A variant Ha-MSV genome (see Fig. 1) was used to generate a virion preparation devoid of helper virus genomes by transfection of the cloned Ha-MSV DNA into ψ 2 cells, which contain a packaging-defective Moloney murine leukaemia virus (Mo-MLV) genome as described by Mann *et al.*⁶. Morphological transformants were selectively cloned and supernatants from cloned cell lines assayed for transforming virus production by *in vitro* focal transformation of NIH 3T3 cells. Three days after seeding, epidermal cells were exposed to ψ 2 cell supernatants containing $\sim 10^6$ Ha-MSV focus-forming units (determined on NIH 3T3 cells); the non-productively infected cells behaved identically to cells productively infected with typical mixtures of Ha-MSV and helper virus particles in culture medium containing either 0.05 mM Ca²⁺ (increased proliferation) or 1.4 mM Ca²⁺ (blocked differentiation)^{4,7}. Unlike cells that were productively transformed, conditioned medium from keratinocyte cultures infected with the ψ 2 preparation did not transform NIH 3T3 cells.

Non-productively infected keratinocytes were removed from culture within 5 days of infection, combined with freshly isolated dermal fibroblasts⁸ and grafted onto prepared skin sites on nude

§ Present address: Hôpital Cochin, Paris, France.

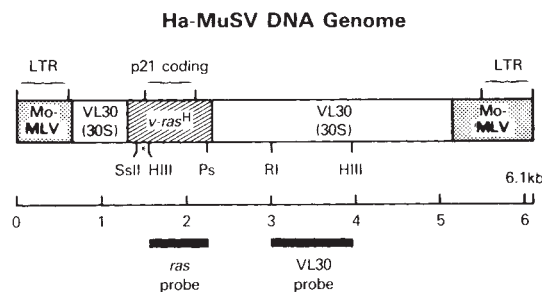


Fig. 1 Organization of the 6.1-kilobase (kb) Harvey murine sarcoma virus genome and location of viral probes. SstII = *SstII*; HIII = *HindIII*; Ps = *PstI*; RI = *EcoRI*. The Ha-MSV genome is derived from at least three different sequences: the rat *c-ras*^H gene, Mo-MLV regulatory sequences and VL30 sequences which are not believed to encode any functional protein¹³. Although the mouse genome contains a *c-ras*^H gene that is homologous to *v-ras*^H, the mouse VL30 sequences are evolutionarily divergent from those of the rat¹⁸ and make the VL30 probe virus-specific. The asterisk indicates where the 0.15-kb *SstII*-*HindIII* fragment of Ha-MSV has been substituted with a corresponding 0.15-kb *EcoRI*-*HindIII* fragment from the rat *c-ras*^H gene¹⁹. This fragment was inserted after an *EcoRI* linker had been added to the viral *SstII* end. This change does not alter the *v-ras*^H coding sequences, but it does increase the titre of the resulting virus by more than one order of magnitude compared with the wild-type viral genome (P.E.T. and D.R.L., unpublished observations).

mice as described previously⁹ with the modifications reported by Worst *et al.*¹⁰ (Fig. 2). Grafts of cultured uninfected keratinocytes produced normal epidermis, but grafts of either BALB/c or Sencar epidermal cells possessing the Ha-MSV genome produced benign tumours after several weeks in virtually all cases where a positive graft take occurred (17 tumours from 23 grafted animals). Histologically, these were typical squamous papillomas indistinguishable from those produced by painting chemical carcinogens and tumour promoters (Fig. 2). Frozen sections of tumours were stained histochemically for γ -glutamyl transpeptidase activity and found to be negative, a result consistent with their histologically benign appearance¹¹.

To determine whether the exogenously transferred Ha-MSV genome was being expressed in the tumours, frozen sections of tumour material were analysed by *in situ* hybridization using ³⁵S-labelled RNA probes (riboprobes) generated in Gemini vectors from two portions of the Ha-MSV genome (Fig. 1). The sections were also studied with probes for two keratin genes which are regulated during epidermal differentiation¹². *In situ* hybridization patterns with the probes for *v-ras*^H and VL30 (rat 30S retrovirus-like sequences, not believed to encode any functional protein¹³) were identical, and expression was exclusively in the epithelial component of tumours (Fig. 3). Transcripts for both genes were detected in the basilar portions of the tumour fronds, but absent in the areas of tumour containing cells in the later stages of differentiation analogous to late spinous cells, granular cells and fully cornified cells in normal skin. Basilar expression varied between regions within the tumours, with some areas showing heavy grain concentrations while grains in adjacent areas were equal to or slightly greater than background level. The more differentiated parts of tumour fronds had consistently low (essentially absent) grain density. Normal epidermis and benign tumours formed by grafting either cultured normal keratinocytes or papilloma cell lines derived from chemically induced tumours¹⁴ did not contain messages detectable by hybridization with either the VL30 or *ras* probe under identical conditions to those used for ψ 2 tumours.

The hybridization pattern with keratin gene probes in ψ 2 tumours (Fig. 3) was similar to that found in benign tumours produced by grafting papilloma cell lines. The transcript of relative molecular mass (*M_r*) 67,000 (67K) was low or absent in the basal cell layer but was very abundant in the first



Fig. 2 Squamous papilloma produced on an athymic nude mouse host by grafting primary mouse keratinocytes containing an exogenous *v-ras*^H oncogene along with normal primary dermal fibroblasts. *a*, Gross appearance of the tumour on the host 3-4 weeks after transplant; *b*, histological section of tumour showing typical squamous papilloma morphology. Section stained with haematoxylin and eosin, magnification was $\times 10$ in the negative.

Methods. Primary newborn BALB/c or Sencar mouse epidermal cells, cultured in Eagle's minimal essential medium containing 0.05 mM Ca²⁺ (ref. 5), were infected on day 3 of culture with a replication-defective variant of Ha-MSV^{6,19} devoid of helper virus genomes, by methods previously used for wild-type Ha-MSV⁷ and at a multiplicity of infection of ~ 1 . After 5 days, cells were removed from culture by treatment with 0.05% trypsin and 0.01% EDTA and combined with homologous primary dermal fibroblasts that had been cultured separately⁸. Combined cell pellets were transplanted onto 8-12-week-old athymic nude mice. All surgery was done under pentobarbital anaesthesia. At 1-3 weeks before transplant, a graft bed was created in host animals by subcutaneous implantation, in the upper back, of a rough glass disk 17 mm in diameter and 3 mm thick¹⁰. Immediately before transplantation, the back skin of the anaesthetized hosts was washed with Betadine, followed by 70% ethanol, an incision was made across the surface of the implanted glass disk, the disk removed and in its place was inserted a thin silicone chamber^{9,10}. Approximately 2×10^6 epidermal cells and 8×10^6 dermal fibroblasts were mixed, centrifuged and pipetted as a cell slurry into each chamber. After cell application to the graft bed, the open chambers were covered with silicone domes and the entire surgical area was protected by a small wire screen shield held in place by four surgical clips to prevent the animals from chewing or otherwise removing the domes⁹. One week after cell application, the screens, domes and implantation chambers were removed and the wound area covered with Adaptic dressing and a gauze bandage. One week later, the bandages and dressing were removed and the wound allowed to heal.

suprabasal cell layer; it remained highly expressed in the maturing portions of the tumour epithelium, consistent with the benign phenotype for these tumours¹⁵. Expression diminished again in the cornified cell region. The 60K keratin gene transcript was expressed in the basal and suprabasal cells of tumour epithelium and diminished only in the most advanced differentiated cells. Neither keratin probe detected messages in the stromal portions of the tumour. In contrast to results with the *ras* and VL30 probes, the hybridization patterns with both keratin probes were uniform in all epithelial areas of the tumours. In *in situ* studies with keratin probes in normal newborn epidermis, transcripts for the 67K keratin gene were detected mainly in suprabasal layers, while the 60K transcripts were present in basal and some suprabasal cells. Neither probe hybridized to cells of the cornified layers.

To determine whether tumours derived from ψ 2-infected keratinocytes contained a functional *v-ras* gene, high-*M_r* DNA isolated from two independent papillomas was transfected into NIH 3T3 cells and scored for transmissible focus-forming activity. These DNAs induced transformed foci of NIH3T3 cells with high efficiency (0.58 foci per μ g DNA), suggesting that the tumours arose from virus-infected epidermal cells inoculated in the prepared graft sites.

These studies clearly indicate that an activated *ras* oncogene is sufficient to produce the benign tumour phenotype in mouse skin. The possibility that other critical changes in the infected keratinocytes occurred as a result of the integration or expression of the exogenous gene cannot be eliminated but seems unlikely,

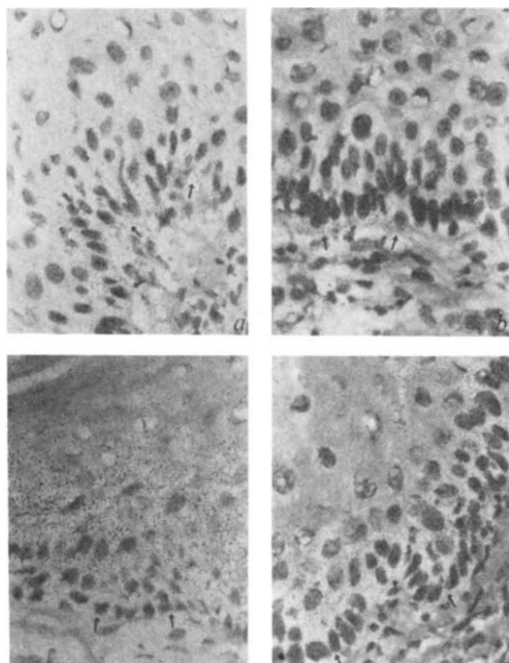


Fig. 3 Detection of *v-ras^H*, VL30 and keratin gene transcripts by *in situ* hybridization. Frozen sections of tumours were hybridized with ³⁵S-labelled RNA corresponding to *v-ras^H* (a), VL30 (b), 67K keratin (c) and 60K keratin (d). Arrows denote stromal-epithelial junctions.

Methods. Tumours were frozen in OCT compound (Miles) and sectioned onto pre-cleaned, autoclaved microscope slides. The sections were air dried for 5 min, fixed in 4% paraformaldehyde in phosphate-buffered saline for 1 min and stored in 70% ethanol at 4 °C until used for hybridization. ³⁵S-labelled RNA probes were transcribed by SP6 or T7 polymerase from Gemini vectors (Promega Biotec) containing the following inserts: *v-ras^H* (a 670-base pair (bp) *Hind*III-*Pst*I fragment containing most of the coding sequence for the p21 *ras* protein; see Fig. 1), VL30 (a 950-bp *Eco*RI-*Hind*III fragment which is virus specific; see Fig. 1), 60K keratin (a 650-bp *Sma*I-*Eco*RI fragment; this gene is expressed in newborn mouse skin, mouse skin tumours and cultured keratinocytes²⁰) and 67K keratin (a 900-bp *Pst*I-*Bam*HI fragment²¹; this gene is expressed in suprabasal cells of normal epidermis and benign tumours but not in chemically induced squamous cell carcinomas¹⁸). Procedures for preparation of RNA probes and *in situ* hybridization as described by Harper *et al.*²². Slides autoradiographed for 2 days and stained with Wright's stain (Harleco).

as the lag time between infection in culture and testing *in vivo* was too short for *in vitro* selection of cells with additional changes. Furthermore, if integration and expression of *v-ras^H* in recipient cells led to further changes in the genotype which were required for tumorigenicity, one would expect selection in tumours for cells with a high copy number and high expression levels, but the *in situ* studies indicated a heterogeneous pattern of expression.

The regulation of *ras* gene transcripts in the differentiated portions of the tumour was surprising considering that the construct inserted contains Mo-MLV long terminal repeat (LTR) regions both 3' and 5' to the *ras* gene coding sequences. A previous study has demonstrated that endogenous MLV LTR transcripts are not expressed in benign mouse skin tumours but are transcribed in malignant tumours¹⁶. Regulation of an exogenous *v-ras^H* gene has been demonstrated independently by Brown *et al.*¹⁷ by inoculating Ha-MSV as a mixed infection with helper virus on mouse skin and promoting with a phorbol ester. Only the basilar portion of the resultant papillomas stained intensely with anti-p21 antibody. In keratinocyte culture, exogenous *v-ras^H* gene expression is not suppressed in differen-

tiating cells⁷, but the oncogene blocks cultured keratinocytes in a late basal cell phenotype⁴. When identical cells are allowed to produce benign tumours *in vivo*, they progress to the later stages of epidermal differentiation where transcripts for the exogenous *v-ras^H* gene are diminished either by a decrease in gene expression or a selective reduction of message stability. It is not clear whether suppression of *v-ras^H* transcription occurs as a primary event in cells receiving a differentiation signal, permitting these cells to proceed to a more advanced differentiation state, or whether the start of advanced differentiation, induced by a strong *in vivo* signal, secondarily suppressed transcription. However, co-expression of *v-ras^H*, VL30 and the 67K keratin messages in the first and second suprabasal cell layers suggests that differentiation begins before the suppression of *v-ras^H* transcription. The regulation of a mutated *c-ras* oncogene by the differentiated portions of benign tumours could account for the benign phenotype in chemically induced tumours where *ras* gene activation is the initiating event. However, studies to test this idea require an analysis of the mutated gene under the influence of its own regulatory sequences.

We thank Anne Kilkenny for cell culture, Dr Thomas Mehrel, Dr Hisayoshi Nakazawa and Christina Cheng for *in situ* hybridization, and Maxine Bellman for typing the manuscript.

Received 9 March; accepted 24 July 1986.

1. Hennings, H. *et al.* *Nature* **304**, 67-69 (1983).
2. Balmain, A., Ramsden, M., Bowden, G. T. & Smith, J. *Nature* **307**, 658-660 (1984).
3. Balmain, A. *Br. J. Cancer* **52**, 1-7 (1985).
4. Yuspa, S. H., Kilkenny, A. E., Stanley, J. & Licht, U. *Nature* **314**, 569-462 (1985).
5. Hennings, H. *et al.* *Cell* **19**, 245-254 (1980).
6. Mann, R., Mulligan, R. C. & Baltimore, D. *Cell* **33**, 153-159 (1983).
7. Yuspa, S. H., Vass, W. & Scolnick, E. *Cancer Res.* **43**, 6021-6030 (1983).
8. Yuspa, S. H., Hennings, H., Dermer, P. & Michael, D. *Cancer Res.* **36**, 947-951 (1976).
9. Yuspa, S. H., Morgan, D. L., Walker, R. J. & Bates, R. R. *Invest. Derm.* **55**, 379-389 (1970).
10. Worst, P. K. M., MacKenzie, I. C. & Fusenig, N. E. *Cell Tissue Res.* **225**, 65-77 (1982).
11. Klein-Szanto, A. J. P., Nelson, K. G., Shah, Y. & Slaga, T. J. *J. natn. Cancer Inst.* **70**, 161-168 (1983).
12. Roop, D. R., Hawley-Nelson, P., Cheng, C. K. & Yuspa, S. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 716-720 (1983).
13. Weiss, R., Teich, N., Varmus, H. & Coffin, J. (eds), *RNA Tumor Viruses. Molecular Biology of Tumor Viruses* 2nd edn. 928-939 (Cold Spring Harbor Laboratory, New York, 1985).
14. Yuspa, S. H. *et al.* *Carcinogenesis* **7**, 949-958 (1986).
15. Toftgard, R., Yuspa, S. H. & Roop, D. R. *Cancer Res.* **45**, 5845-5850 (1985).
16. Housey, G. M. *et al.* *Biochem. biophys. Res. Commun.* **1**, 391-398 (1985).
17. Brown, K. *et al.* *Cell* **46**, 447-456 (1986).
18. Ellis, R. W. *et al.* *J. Virol.* **36**, 408-420 (1980).
19. DeFeo, D. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 3328-3332 (1981).
20. Steinert, P. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 5709-5713 (1984).
21. Steinert, P. M. *et al.* *J. biol. Chem.* **260**, 7142-7149 (1985).
22. Harper, M. E., Marselle, L. M., Gallo, R. C. & Wong-Stahl, F. *Proc. natn. Acad. Sci. U.S.A.* **83**, 772-776 (1986).

Unusual priming mechanism of RNA-directed DNA synthesis in *copia* retrovirus-like particles of *Drosophila*

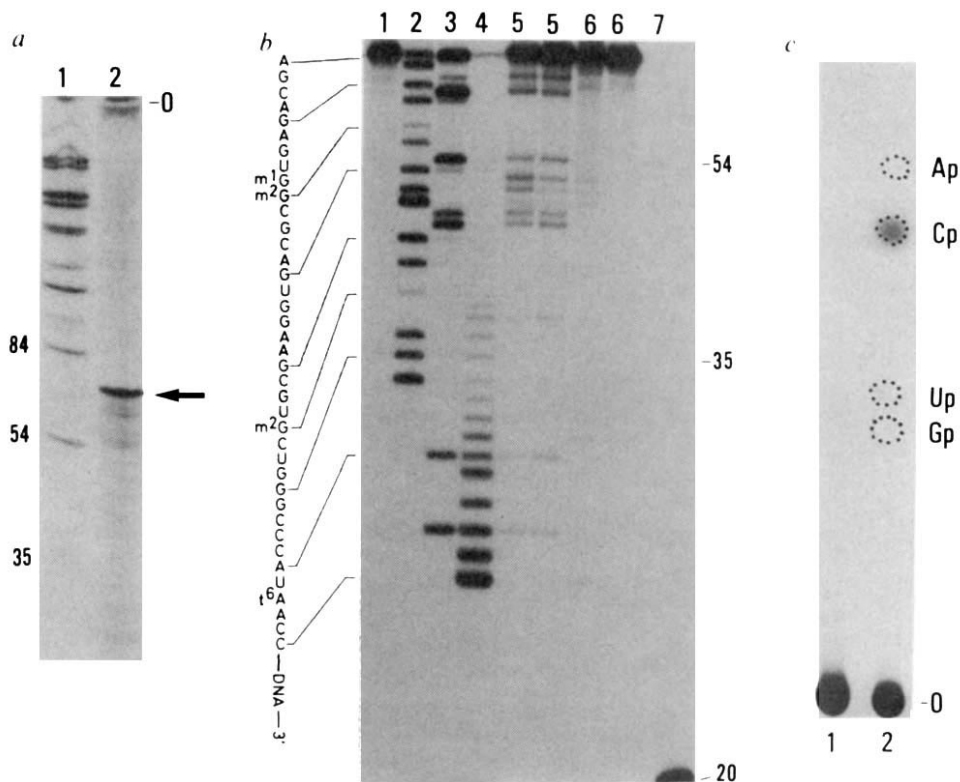
Yo Kikuchi*, Yumiko Ando* & Tadayoshi Shiba†‡

* Laboratory of Molecular Genetics and † Laboratory of Cell Biology, Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan

Drosophila cells contain virus-like particles (VLPs) containing 5 kilobases (kb) of RNA (VLP H-RNA) homologous to the transposable element *copia*¹. The identity between VLP H-RNA and *copia* DNA has previously been confirmed at the nucleotide sequence level² and reverse transcriptase activity is also detected in the VLPs¹. These results suggest that VLPs and *copia* are derivatives of viral particle and provirus forms, respectively, of the *copia* retrovirus-like particle. If the *copia* retrovirus-like particle replicates by a mechanism similar to the mechanism of vertebrate

‡ Present address: Laboratory of Molecular and Cellular Biology, Kitasato University, School of Hygienic Science, 1-15-1 Kitasato, Sagami-hara-shi, Kanagawa 228, Japan.

Fig. 1 Isolation and characterization of the limited primer extension product using VLP RNAs as a template/primer. Using the endogenous RNA primer, DNA was synthesized *in vitro* with reverse transcriptase from AMV in the presence of ddCTP (*a*), then the RNA moiety of the product was sequenced (*b*), and the nucleotides of the RNA-DNA junction were determined (*c*). *a*, Electrophoretic analysis of the limited primer extension product labelled with [α - 32 P]dATP. Lanes: 1, size markers are Φ X174 RF DNA *Hha*I fragments labelled with [γ - 32 P]ATP and T4 polynucleotide kinase; 2, the reaction product. Arrow, 64-nucleotide-long major band; numbers, sizes (bases) of marker fragments. *b*, Sequence analysis of the RNA moiety of the product. Lanes: 1, no enzyme added; 2, 3, 5, 6, partial digest products with RNase T₁ (G), U₂(A>G), PhyM(A+U), and *Bacillus cereus* RNase(C+U), respectively; 4, controlled alkaline hydrolysis; 7, size markers as in *a*. The size marker 20 nucleotides in length is a *Taq*I fragment of Φ X174 RF DNA labelled as described in *a*. Repeated sequencing analyses (not shown) established unequivocally the nucleotide sequence (except modified bases as shown in the left side of the autoradiogram). *c*, Analysis of the nucleotides at the RNA-DNA junction of the product. The limited primer extension product using ddCTP was labelled with [α - 32 P]dTTP. The product was digested with RNase T₂ and analysed by thin-layer chromatography. Lanes: 1, no enzyme added; 2, treated with RNase T₂. Dotted circles indicate the authentic ribonucleoside 3'-phosphates (Ap, Cp, Up and Gp). RNase T₂ digestion of [α - 32 P]dTTP-labelled product yielded [32 P]Cp (lane 2) indicating that the RNA-DNA junction of this limited extension product has the sequence 5'-rCpdT-3'.



Dotted circles indicate the authentic ribonucleoside 3'-phosphates (Ap, Cp, Up and Gp). RNase T₂ digestion of [α - 32 P]dTTP-labelled product yielded [32 P]Cp (lane 2) indicating that the RNA-DNA junction of this limited extension product has the sequence 5'-rCpdT-3'.

Methods. *a*, VLP and VLP RNAs were prepared from cultured GM₂ cells as described previously¹. The mixture (15.6 μ l) containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM MgCl₂, 5 mM dithiothreitol and 5 μ g VLP RNAs was incubated at 65 °C for 5 min, then at 42 °C for 30 min. We then added 2.4 μ l of 1.7 mM each dGTP, dTTP and ddCTP and 1 μ l [α - 32 P]dATP (total 10 μ Ci, 3,000 Ci mmol⁻¹) and 1 μ l (12.6 U) AMV reverse transcriptase (Seikagaku Kogyo, Tokyo) to the mixture, and the incubation was continued for 1 h. The reaction was terminated by heating at 95 °C for 2 min. The nucleic acid was precipitated by ethanol and electrophoresed in 20% polyacrylamide/8 M urea gel (0.05 $\times$ 12 $\times$ 17 cm) using 90 mM Tris-borate/2 mM EDTA buffer (pH 8.3). Labelled products were detected by autoradiography. *b*, The 64-nucleotide-long product (lane 2 in *a*) was eluted from the gel and sequenced using the base-specific RNase method, essentially as described in refs 15-17. *c*, The limited extension product using ddCTP was obtained from the reaction as described in *a*, except that [α - 32 P]dTTP instead of [α - 32 P]dATP was used. The product eluted from the gel was digested with RNase T₂, and chromatographed on a cellulose thin-layer plate (solvent: isobutyric acid/concentrated ammonia/H₂O = 577:38:385, v/v/v). The 32 P-labelled nucleotide was detected by autoradiography.

retroviral replication, a cellular transfer RNA would prime synthesis of the first DNA strand. We show that this is indeed so but that *copia* retrovirus-like particle has a novel type of priming mechanism; the first DNA extension does not start from the 3' end of a tRNA, but from an internal site (two nucleotides after the anticodon loop) of the *Drosophila* initiator methionine tRNA (tRNA_i^{Met}).

Retroviruses, a family of eukaryotic RNA viruses, replicate through a DNA intermediate. RNA-directed DNA synthesis is catalysed by a primer-dependent reverse transcriptase, similar to other known DNA polymerases. The replication of all retroviruses so far examined is initiated by covalent addition of a deoxynucleotide to the 3' end of a cellular tRNA molecule that is hydrogen-bonded to the primer binding site (PBS) of the viral genome RNA³. Primer tRNAs so far identified are tryptophan tRNA, proline tRNA and lysine tRNA for the avian sarcoma and leukemia virus groups, the murine leukaemia viruses and the mouse mammary tumour virus group, respectively⁴⁻⁹. In other retrovirus-like systems, phenylalanine tRNA, glycine tRNA and glutamine tRNA were also proposed as primers, because the 3' terminal sequences of these tRNAs are complementary to the putative PBSs on the genomes¹⁰⁻¹³. Recently, Inouye *et al.* cloned the gene encoding *Drosophila* serine tRNA using a synthetic oligonucleotide probe with the sequence identical to the putative PBS of *Drosophila* retrotransposon 297¹⁴. Because the 3' terminal 18 nucleotides of this serine tRNA are complementary to the putative PBS of 297, this serine tRNA was proposed as a potential primer of 297. But, so far, no primer

tRNA species has been proposed in *Drosophila copia* VLP.

In previous analyses of *Drosophila* VLP RNAs, it was shown that the VLP contained various species of tRNAs in addition to the VLP H-RNA¹. One of these tRNAs could be used as a primer for RNA-directed DNA synthesis in the VLP. Probably because of the low activity of reverse transcriptase in VLPs, attempts to synthesize the primer extension product using VLP itself as an enzyme source were unsuccessful. We used reverse transcriptase from avian myeloblastosis virus (AMV) as an enzyme for the VLP RNA-directed DNA synthesis. To identify the primer tRNA in the VLP, we incubated the VLP RNAs with [α - 32 P]dATP, dTTP, dGTP, dideoxy CTP (ddCTP) and AMV reverse transcriptase as described in Fig. 1 legend. Using this mixture, and fractionation in a 20% polyacrylamide 8 M urea gel (Fig. 1*a*, lane 2), we detected a major single band 64 nucleotides in length. Although the size of this product was much smaller than the size of tRNA, this molecule was expected as the limited extension product from an RNA primer. Because this product was a 3' end 32 P-labelled RNA, it was possible to analyse the RNA moiety of this product by the enzymatic RNA sequencing procedure; involving partial digestion with base-specific RNases¹⁵⁻¹⁷. As shown in Fig. 1*b*, the shortest digestion fragment of the ladder is ~25 nucleotides long. This indicates that the product consists of an ~25-nucleotide-long DNA molecule (resistant to digestion) at its 3' end and a 39-nucleotide-long RNA at its 5' end and that the RNA moiety was used as a primer for limited DNA extension using ddCTP. The sequence of the RNA moiety of this product corresponds to the published



Fig. 2 Priming model for *copia* retrovirus replication. Upper two lines, the genome organizations of *copia* DNA and RNA. The region near the edge of the 5' LTR is enlarged to show its nucleotide sequence. The sequence has been determined by Emori *et al.*² and is shown here as a DNA sequence. The 15 nucleotides next to the 5' LTR are complementary to the 15 nucleotides (25-39) of *Drosophila* tRNA_{Met}. The complete nucleotide sequence of tRNA_{Met} taken from the data of Silverman *et al.*¹⁸ is represented in cloverleaf form. The sequence of nucleotides 1-39 of this tRNA is consistent with the analysis of the RNA moiety of the limited primer extension product, as shown in Fig. 1b.

sequence, nucleotides 1–39, of *Drosophila* tRNA_i^{Met} (ref. 18). The surprising result shows that DNA extension does not occur at the 3' end of the tRNA but at the internal site of tRNA_i^{Met}. Although we did not extensively analyse the modified bases because of the limited amount of this product, information obtained from the autoradiogram of the RNA sequencing gel (Fig. 1b) suggests the presence of the same modified bases as in the published sequence of tRNA_i^{Met}. *N*-((9-β-D-ribofuranosyl purine-6-yl)carbamoyl)threonine(t⁶A) and 1-methylguanosine(m¹G) were reported to be resistant to RNase U₂ and RNase T₁, respectively, thus giving no bands, and also reported to give increased distances between bands in the ladder from partial alkaline hydrolysis¹⁷. Also, *N*-2-methylguanosines(m²G) are known to give weak bands in RNase T₁ partial digestion because m²G is moderately resistant to RNase T₁ (ref. 17). The RNA sequencing ladder observed allows us to conclude that the RNA moiety of the product constitutes nucleotides 1–39 of tRNA_i^{Met}.

The 15 nucleotides, 25–39, of tRNA^{Met}_i are complementary to the region just after the left-hand long terminal repeat (5' LTR) of VLP H-RNA (Fig. 2). If ddCTP was added to the primer extension reaction mixture, DNA synthesis would stop

at the first G residue on the template. The first G residue is located 25 nucleotides upstream from the 3' end of the LTR (Fig. 2 and ref. 2). As seen in Fig. 1, the DNA moiety of our limited extension product was 25 nucleotides long, consistent with the size predicted from the model shown in Fig. 2. To confirm the location of priming, we identified the nucleotides at the RNA-DNA junction of the product. As shown in Fig. 1c, the junction sequence was determined as 5'-rCpdT-3', which is also consistent with the priming site postulated in the model (Fig. 2). We conclude that the VLP uses half of the tRNA^{Met} molecule (nucleotides 1-39) as a primer for RNA-directed DNA synthesis and that the PBS of VLP H-RNA is located just after 5'LTR without any nucleotide inserts between the 5' LTR and PBS (Fig. 2).

In vertebrate retroviruses, reverse transcription of the viral RNA yields a circular provirus containing two LTRs that are 4 base pairs (bp) longer than the integrated provirus³. These four nucleotides are located at the junction between the two LTRs. In the reverse transcription model, two of these four bp are derived from the nucleotides between the 5' LTR and PBS³. In the *copia* system, Flavell and Ish-Horowitz isolated seven two-LTR *copia* circles and determined the LTR-LTR junction sequences of these circles¹⁹. They reported that one of these circles contained perfectly fused repeats with no nucleotide insert between the two LTRs. Moreover, Flavell and Brierley recently reported that this type of circle comprises 50% of extrachromosomal circular *copias* and that the termini of extrachromosomal linear *copias* are identical to those of genomic *copias*²⁰. As there is no nucleotide insert between the 5' LTR and PBS in *copia* as described above, these molecules can now be thought to be generated from the reverse transcription of VLP H-RNA and to be circular and linear DNA proviruses.

As yet we do not know how tRNA_i^{Met} is cleaved at the specific site (between nucleotides 39 and 40) or by which enzyme. As this site is located in the stable G+C-rich anticodon stem in intact tRNA_i^{Met}, it is most probable that tRNA_i^{Met} is cleaved by single-strand-specific 3' → 5' exoribonuclease after hybridization of tRNA_i^{Met} to the PBS of the RNA genome. Ribonuclease activities in retroviruses and reverse transcriptase have been reported²¹ and these activities could be candidates for the specific enzymatic cleavage of tRNA_i^{Met}.

We have no reason to exclude the possibility that the new priming mechanism described here occurs in retroviruses other than *copia*. Thus, in investigating retroviral systems, we should consider not only a TGG sequence complementary to the tRNA 3' end but also several nucleotides next to the 5' LTR as a putative PBS.

We thank Drs T. Miyake and F. Hishinuma for critical discussions and encouragement, S. Togashi for providing VLPs and M. Tsuchiya-Mikuni for assistance.

Received 9 June; accepted 17 July 1986.

1. Shiba, T. & Saigo, K. *Nature* **302**, 119-124 (1983).
2. Emori, Y. *et al.* *Nature* **316**, 773-776 (1985).
3. Varmus, H. & Swanstrom, R. in *Molecular Biology of Tumor Viruses* 2nd edn RNA Tumor Viruses (eds Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 369-512 (Cold Spring Harbor Laboratory, New York, 1982).
4. Dahlberg, J. E. *et al. J. Virol.* **13**, 1126-1133 (1974).
5. Harada, F., Sawyer, R. C. & Dahlberg, J. E. *J. biol. Chem.* **250**, 3487-3497 (1975).
6. Harada, F., Peters, G. G. & Dahlberg, J. E. *J. biol. Chem.* **254**, 10979-10985 (1979).
7. Peters, G. *et al. J. Virol.* **21**, 1031-1041 (1977).
8. Taylor, J. M. *Biochim. biophys. Acta* **473**, 57-71 (1977).
9. Peters, G. & Glover, C. *J. Virol.* **35**, 31-40 (1980).
10. Ono, M. & Ohishi, H. *Nucleic Acids Res.* **11**, 7169-7179 (1983).
11. Hodgson, C. P., Elder, P. K., Ono, T., Foster, D. N. & Getz, M. J. *Molec. cell. Biol.* **3**, 2221-2231 (1983).
12. Ou, C., Boone, L. R. & Yang, W. K. *Nucleic Acids Res.* **11**, 5603-5620 (1983).
13. Colicelli, J. & Goff, S. P. *J. Virol.* **57**, 37-45 (1986).
14. Inouye, S., Saigo, K., Yamada, K. & Kuchino, Y. *Nucleic Acids Res.* **14**, 3031-3043 (1986).
15. Donis-Keller, H., Maxam, A. M. & Gilbert, W. *Nucleic Acids Res.* **4**, 2527-2538 (1977).
16. Donis-Keller, H. *Nucleic Acids Res.* **8**, 3133-3142 (1980).
17. Krupp, G. & Gross, H. J. in *The Modified Nucleotides in Transfer RNA II: A Laboratory Manual of Genetic Analysis, Identification and Sequence Determination* (eds Agris, P. F. & Kopper, R. A.) 11-58 (Liss, New York, 1983).
18. Silverman, S. *et al. Nucleic Acids Res.* **6**, 421-433 (1979).
19. Flavell, A. J. & Ish-Horowitz, D. *Cell* **34**, 415-419 (1983).
20. Flavell, A. J. & Brierley, C. *Nucleic Acids Res.* **14**, 3659-3669 (1986).
21. Hung, P. P. *Virology* **51**, 287-296 (1973).

Phosphorylation of tubulin enhances its interaction with membranes

Alan J. Hargreaves, Francisco Wandosell & Jesús Avila

Centro de Biología Molecular (CSIC-UAM),
Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Tubulin, the main component of intracellular microtubules, is also a major protein in subcellular membrane preparations¹⁻⁴ and can interact with biological⁵⁻⁷ and artificial⁸⁻¹² membranes *in vitro*. Of particular interest is the association of tubulin with postsynaptic junctional lattices enriched in a polypeptide of relative molecular mass (M_r) 50,000 (50K)¹³, recently identified as the major subunit of the calmodulin-dependent protein kinase¹⁴. Phosphorylation of tubulin with a calmodulin-dependent protein kinase similar to that found in postsynaptic densities inhibits its ability to self-assemble into microtubules in a reversible fashion¹⁵. This involves the phosphorylation of residues in its 4K carboxy-terminal region, a domain that seems to regulate its self-assembly¹⁶⁻¹⁸. The results presented here suggest that the phosphorylation of tubulin with this kinase enhances its ability to interact with membranes. This effect is reversible.

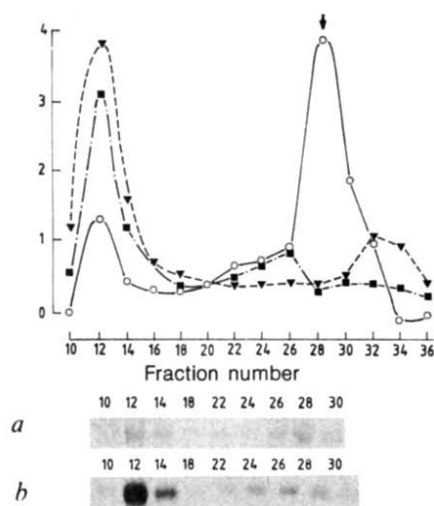


Fig. 1 Analysis of fractions collected from Sepharose 4B-CL chromatography of tubulin-phospholipid mixtures. *a*, Coomassie blue-stained protein of even-numbered fractions (only the region of the tubulin band is shown); *b*, autoradiography of the same gel. **Methods.** L- α -phosphatidyl choline ($\sim 100 \mu\text{g}$) from bovine brain (Sigma) was dried under N_2 and stored for 1 h in a vacuum dessicator before each experiment. Tubulin ($100 \mu\text{g}$), purified from pig brain by two cycles of temperature-dependent assembly and disassembly²³ followed by phosphocellulose chromatography¹⁹, was phosphorylated with calmodulin-dependent protein kinase¹⁵ in $100 \mu\text{M}$ ATP and $^{32}\text{P}[\gamma]\text{ATP}$ (Amersham), in a final volume of 0.2 ml . Under these conditions 0.4 mol phosphate was incorporated per mol tubulin dimer. Phosphorylated tubulin was then mixed with a fourfold excess of non-phosphorylated tubulin and the mixture was gently sonicated (under N_2) together with the dried phospholipid extract in an MSE sonicator. The sonicate was then diluted 1:4 with column buffer (10 mM Mes, 100 mM NaCl, 6 mM KCl, pH 6.4) and applied to a Sepharose 4B-CL column ($20 \times 1 \text{ cm}$) equilibrated with the same buffer. Fractions of 0.5 ml were collected and their optical density at 260 nm ($\blacktriangledown$) and their protein content²⁰ ($\circ$) were measured. Aliquots were then analysed by electrophoresis in a 7% polyacrylamide gel overlaid with a 4% stacking gel, followed by autoradiography using X-ray film. Bands visualized by autoradiography were cut out and their Cerenkov radiation (black squares) measured in an Intertechnique liquid scintillation counter. Arrow, elution position for tubulin in the absence of vesicles. Units: $\blacktriangledown$, $\text{OD}_{260} \times 10$; $\circ$, $A_{595} \times 10$; $\blacksquare$, c.p.m. $\times 0.01$.

Table 1 Interaction of tubulin with vesicles

Phosphatase	Vesicle-bound protein (pellet)		Released protein (super)	
	^{32}P	^3H	^{32}P	^3H
–	95	100	<2	<1
+	55	70	40	30

Data are % initial radioactive content in the tubulin-vesicle complex before phosphatase treatment. Total initial radioactivity, $4,750 \text{ c.p.m.}$ for ^{32}P and $3,000 \text{ c.p.m.}$ for ^3H in each sample.

We studied the behaviour of tubulin in the presence of the neutral phospholipid phosphatidyl choline, because this interaction has been well characterized⁸⁻¹². Purified tubulin¹⁹ was phosphorylated with the calcium- and calmodulin-dependent protein kinase as described by Wandosell *et al.*¹⁵ and gently sonicated in the presence of phosphatidyl choline. The mixture was then chromatographed on a Sepharose 4B-CL column and the eluted fractions analysed by gel electrophoresis, autoradiography and protein assay²⁰ (Fig. 1). Although most of the ^{32}P -labelled tubulin was detected in the void volume fractions, a large proportion of total unlabelled tubulin, as determined by staining with Coomassie blue or by protein assay, was included. Because, in the absence of vesicles, both phosphorylated and non-phosphorylated tubulin are normally detected in the Sepharose 4B fractions¹⁵ (Fig. 1, arrow) and the phospholipid vesicles (measured by light scattering at 260 nm) elute in the void volume, this result suggests that phosphorylated tubulin has a greater capacity to interact with membranes than does non-phosphorylated tubulin.

To confirm this suggestion, we performed a reversal experiment. Tubulin was labelled in the carboxy-terminal end of the α -subunit with tritiated tyrosine, phosphorylated and mixed with phospholipid vesicles as described above. The (^{32}P - ^3H) tubulin-vesicle complexes eluting in the void volume of the Sepharose 4B-CL column were then incubated in the presence or absence of alkaline phosphatase before the liberated and vesicle-bound tubulin were separated by centrifugation (Fig. 2, Table 1). We found that, although $\sim 40\%$ of the tubulin-bound phosphate was removed by phosphatase treatment, 30% of the tyrosinated tubulin was released from the vesicles, which strongly suggests that the interaction of phosphorylated tubulin with membranes is reversible.

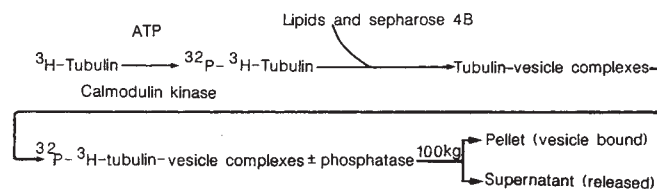


Fig. 2 Reversal of the interaction of phosphorylated tubulin with vesicles by phosphatase treatment. Tubulin in the total soluble protein fraction from pig brain was tyrosinated with tritiated tyrosine under the conditions of Arce *et al.*²⁴, and subsequently purified as indicated in Fig. 1 legend. This preparation incorporated $\sim 0.01 \text{ mol}$ tyrosine per mol tubulin dimer. ^3H -tubulin ($50 \mu\text{g}$) was phosphorylated and mixed with phospholipid vesicles as described in Fig. 1: tubulin incorporated 0.8 mol phosphate per mol protein. The (^{32}P - ^3H) tubulin-vesicle complexes eluting in the void volume of the Sephadex 4B-CL column were pooled, made up to 50 mM with Tris, pH 7.5, and incubated $\pm 10 \text{ U ml}^{-1}$ alkaline phosphatase (Sigma) for 20 min at 30°C . The samples were then centrifuged at $100,000 \text{ g}$ for 15 min in a Beckman airfuge and then protein in the supernatant (released) or pellet (vesicle-associated) was precipitated with 10% trichloroacetic acid. Tritium and ^{32}P radioactivity were then determined for each fraction, after the addition of scintillation fluid using an Intertechnique (Kontron) liquid scintillation counter. In the case of liberated inorganic phosphate, total c.p.m. were measured in the supernatant. In control experiments in the absence of vesicles, phosphorylated tubulin did not sediment under the same centrifugation conditions.

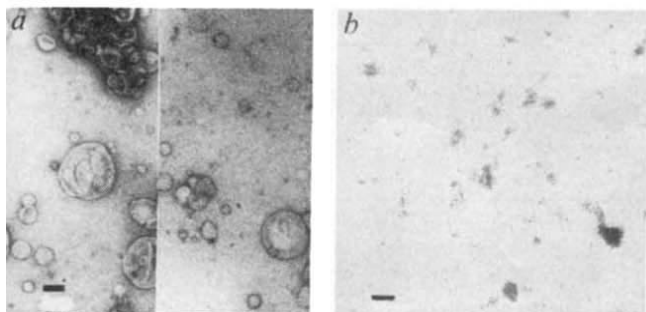


Fig. 3 Characterization of vesicle-tubulin complexes by direct negative staining and immunoelectron microscopy. *a*, Composite including all types of structure visualized; *b*, area of the specimen grid containing small vesicles to allow clear visualization of gold labelling. Bar, 0.1 μ m.

Methods. Tubulin was phosphorylated and sonicated in the presence of phospholipid as described in Fig. 1, except that non-phosphorylated tubulin was not added. Aliquots of fractions eluted in the void volume of the Sepharose 4B-CL column were applied directly to carbon and collodion-coated 300-mesh copper grids and either (*a*) stained directly with 1% uranyl acetate or (*b*) fixed for analysis by immunoelectron microscopy. For the latter, a 200- μ l sample was fixed for 10 min at room temperature in the presence of 0.25% w/v glutaraldehyde. Fixation was stopped by the addition of 0.4% w/v glycine and the sample then adsorbed onto the specimen grid and incubated for 5 min with column buffer containing 10% ovalbumin before being incubated for 30 min with a rabbit anti-tubulin serum⁴. It was then washed extensively with column buffer before being incubated with protein A-gold complexes²¹ for 30 min. Samples were washed extensively with column buffer, stained weakly with 0.5% uranyl acetate and examined in a Jeol 100B electron microscope.

To analyse the nature of the liposomes containing associated tubulin, aliquots of these void-volume fractions were applied to carbon- and collodion-coated copper grids, stained directly with 1% aqueous uranyl acetate and examined in a Jeol 100B electron microscope. We observed various vesicle sizes (Fig. 3*a*), together with fused membranes and vesicles aggregates similar to those observed elsewhere⁸⁻¹². To ensure that the vesicle-associated tubulin was adsorbed onto membrane structures, and was not caused by non-specific aggregation, we incubated samples with a rabbit anti-tubulin serum⁴ followed by protein A-gold complexes²¹. As shown in Fig. 3*b*, the gold balls preferentially associated with vesicle structures, suggesting that tubulin interacts with the membranes and that at least some of its antigenic determinants are exposed on the membrane surface. Control samples, containing vesicles without tubulin, show little labelling with gold particles (data not shown).

As mentioned above, tubulin is phosphorylated at the 4K carboxy-terminal-containing domain under our experimental conditions. To determine whether the phosphorylated 4K domain is directly involved in the interaction with membranes, Sepharose 4B-CL fractions containing ³²P-tubulin associated with vesicles were incubated in the presence of 1% w/w subtilisin under conditions that selectively remove this fragment¹⁷. Although most digested protein stained by Coomassie blue was composed of a polypeptide doublet of *M_r* ~50K, the ³²P label disappears completely after proteolytic digestion (A.J.H., unpublished data). This finding indicates that the carboxy-terminal region of vesicle-associated ³²P-tubulin is accessible to proteolysis and, therefore, not directly involved in the interaction with membranes.

Because hydrophobic interactions are involved in the insertion of tubulin into lipid bilayers¹⁰⁻¹⁴, it is possible that phosphorylated tubulin is more hydrophobic than the non-phosphorylated form. Indeed, when phosphorylated tubulin was analysed by charge-shift electrophoresis under the conditions of Helenius and Simons²², there is an increase in its electrophoretic migration

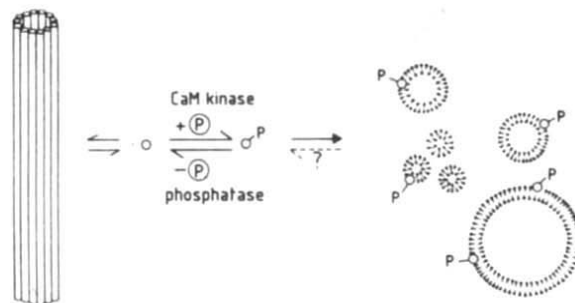


Fig. 4 Model for the regulation of tubulin distribution between microtubules and membranes by calmodulin-dependent protein kinase. Two distinct pools of tubulin dimers (○) are illustrated. Dimers phosphorylated by the protein kinase probably incorporated into membranes and do not polymerize into microtubules, whereas non-phosphorylated tubulin dimers interact to a lower extent with membranes and can self-assemble.

in the presence of charged detergents compared with non-phosphorylated controls (not shown). This result is consistent with the notion that the conformational change induced by phosphorylation of tubulin with calmodulin-dependent protein kinase reveals hydrophobic domains that interact with membranes. A similar post-translational modification, regulated by kinase/phosphatase action, could be involved in the insertion of newly synthesized tubulin into microsomal membranes⁷ and therefore might act as a fine-control mechanism of the intracellular distribution of tubulin. As suggested in Fig. 4, the dimers in the soluble tubulin pool may be able to adopt two distinct conformations. One, induced by phosphorylation with the calmodulin-dependent protein kinase, could facilitate the insertion into membranes, whereas in the second, the non-phosphorylated dimers would be available for polymerization into microtubules. Because the 4K carboxy-terminal region of tubulin can regulate microtubule self-assembly and its phosphorylation enhances the association of tubulin with membranes, this domain may be fundamental in the regulation of microtubule- and membrane-based events.

Project supported by a grant from the Comisión Asesora para el Desarrollo de la Investigación Científica y Técnica. F.W. is a postdoctoral fellow of the C.S.I.C.

Received 3 June, accepted 31 July 1986.

- Marotta, C. A., Stocchi, P. & Gilbert, J. M. *Brain Res.* **167**, 93-106 (1979).
- Zisapel, N., Levi, N. & Gozes, I. *J. Neurochem.* **45**, 490-496 (1980).
- Estridge, M. *Nature* **268**, 60-63 (1977).
- Hargreaves, A. J. & Avila, J. J. *J. Neurochem.* **45**, 490-496 (1985).
- Soifer, D. & Czosnek, H. *J. Neurochem.* **35**, 1128-1136 (1980).
- Bernier-Valentin, F. & Rousset, B. *J. biol. Chem.* **257**, 7092-7099 (1982).
- Bernier-Valentin, F., Aunis, D. & Rousset, B. *J. Cell Biol.* **97**, 209-216 (1983).
- Caron, J. M. & Berlin, R. D. *J. Cell Biol.* **81**, 665-671 (1979).
- Berlin, R. D., Caron, J. M., Huntley, R., Melmed, R. N. & Oliver, J. M. in *Biological Functions of Microtubules and Related Structures* (eds Sakai, M., Mohri, H. & Borisy, G. G.) 405-423 (Academic, New York, 1982).
- Klausner, R. D., Kumar, N., Weinstein, J. N., Blumenthal, R. & Flavin, M. *J. biol. Chem.* **256**, 5879-5885 (1981).
- Kumar, N., Klausner, R. D., Weinstein, J. N., Blumenthal, R. & Flavin, M. *J. biol. Chem.* **256**, 5886-5889 (1981).
- Kumar, N., Blumenthal, R., Henkart, M., Weinstein, J. N. & Klausner, R. D. *J. biol. Chem.* **257**, 15137-15144 (1982).
- Kennedy, M. B., Bennett, M. K. & Erondy, N. E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7357-7361 (1983).
- Walters, B. B. & Matus, A. I. *Nature* **257**, 496-498 (1975).
- Wandosell, F., Serrano, L., Hernández, M. A. & Avila, J. *J. biol. Chem.* **261**, 10332-10339 (1986).
- Serrano, L., Avila, J. & Maccioni, R. B. *Biochemistry* **23**, 4675-4681 (1984).
- Serrano, L., de la Torre, J., Maccioni, R. B. & Avila, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5985-5993 (1984).
- Serrano, L., Montejo de Garcini, E., Hernández, M. A. & Avila, J. *Eur. J. Biochem.* **153**, 595-600 (1986).
- Weingarten, M., Lockwood, A., Hwo, S. & Kirschner, M. W. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1858-1862 (1975).
- Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).
- Roth, J. *Tech. Immunochim.* **1**, 108-133 (1982).
- Helenius, A. & Simons, K. *Proc. natn. Acad. Sci. U.S.A.* **74**, 529-532 (1977).
- Shelanski, M. L., Gaskin, F. & Cantor, C. R. *Proc. natn. Acad. Sci. U.S.A.* **70**, 765-768 (1973).
- Arce, C. A., Rodriguez, J. A., Barra, H. & Caputto, R. *Eur. J. Biochem.* **59**, 145-149 (1975).

Receptor modulation in brain slices

E.W. Karbon and S.J. Enna

Brain tissue preparations provide a window on the regulation of neuroreceptor function that cell-free preparations cannot offer. The view reveals complex neuromodulator interactions.

MANY neurotransmitters influence neuronal function by directly affecting either of two important signal transduction mechanisms. Of these mechanisms, the most well-characterized, is the receptor-coupled adenylate cyclase system¹. Stimulation of the cyclase enzyme enhances the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), while inhibition decreases cAMP production. The cyclic nucleotide then acts as a "second messenger" by stimulating a protein kinase that phosphorylates selected proteins.

The other important receptor-coupled effector system is phosphatidylinositol turnover². In response to receptor activation, phospholipase C degrades phosphatidylinositol 4,5-bisphosphate, a phospholipid, to dual second messengers. One messenger, inositol triphosphate, liberates calcium ions from internal stores; the other, diacylglycerol, stimulates protein kinase C that, like the cAMP-dependent enzyme, phosphorylates multiple protein substrates.

The ability of neurotransmitters to influence these processes has routinely been examined in brain slices, intact tissue preparations that closely approximate *in vivo* conditions. These slices are maintained in an aerated, physiological buffer and are pre-labelled with a substrate precursor such as ³H-adenine, which is converted to ³H-ATP, or ³H-inositol, which is incorporated into phospholipid pools. Enzyme activity in response to receptor activation can then be assessed by measuring the levels of radiolabelled product that have accumulated³.

Mixed reception

Given the importance of identifying the events which follow receptor activation, the brain slice preparation is a valuable tool. Recent studies have shown that, although the direct stimulation of second messenger formation by neurotransmitters is often sufficient to account for the biochemical effects they provoke, many neurotransmitter receptors are also subject to the regulatory influences of compounds acting at distinct receptor sites. Such compounds are called "neuromodulators" since they indirectly modify the biochemical response resulting from activation of another receptor.

For example, brain slices exposed to noradrenaline, which stimulates both α - and β -adrenergic receptors, show a greater

accumulation of cyclic AMP than those treated with isoprenaline, a selective β -adrenergic agonist^{4,5} (Table 1). These results suggest that α -adrenergic receptors, like the β -adrenergic components, may be directly coupled to adenylate cyclase. But α -agonists such as 6-fluoronoradrenaline (6-FNA) do not of themselves stimulate cAMP accumulation (Table 1).

Table 1 Neuromodulator effects on cAMP accumulation

	cAMP accumulation (% conversion)	
	Basal	Isoprenaline
No addition	0.06	0.38
6-FNA (10 μ M)	0.07	0.77
Baclofen (50 μ M)	0.11	1.14
PDBu (10 μ M)	0.06	1.20
Noradrenaline (100 μ M)	0.80	—

The influence of various agents on cAMP accumulation in rat brain cerebral cortical slices. Results are expressed as per cent conversion, which represents the percentage of total tritium present as ³H-cyclic AMP. 6-FNA, 6-fluoronoradrenaline; baclofen, β -p-chlorophenyl γ -aminobutyric acid; PDBu, phorbol 12, 13-dibutyrate. (Adapted from refs 3,7,9.)

When, however, rat brain slices are exposed to both 6-FNA and isoprenaline, the cAMP response equals that obtained when noradrenaline alone is used. These findings can be taken as evidence that α -adrenergic receptors are indirectly coupled to the adenylate cyclase system and that noradrenaline, through interaction with these sites, can function as a neuromodulator.

Similar results have recently emerged from studies using the agonist β -p-chlorophenyl γ -aminobutyric acid (baclofen). Baclofen specifically recognizes a γ -aminobutyric acid (GABA) receptor subtype which is referred to as the GABA_B site⁶. Like 6-FNA, baclofen augments cAMP accumulation markedly in brain slices following exposure to a number of β -adrenergic receptor stimulants, while being ineffective alone in this regard⁷ (Table 1). The augmenting response elicited by both GABA_B and α -adrenergic agonists is independent of phosphodiesterase activity and totally dependent upon the presence of extracellular calcium, suggesting a common mechanism of action^{3,8}.

The ability to augment cyclic AMP formation is not limited to endogenous substances, for it can be elicited by phorbol esters, compounds that mimic the action of diacylglycerol and activate protein kinase C^{9,10} (Table 1). This finding is intri-

guing in that it suggests that activation of phospholipase C and the subsequent generation of diacylglycerol may be the mechanism responsible for the augmentation observed in response to GABA_B and α -adrenergic receptor activation. Such a mechanism would point to a positive interaction between two major signalling pathways¹¹.

A cut above

Brain slice preparations have been invaluable in elucidating a role for α -adrenergic and GABA_B receptors, because the augmentation phenomenon is not observed in cell-free preparations. The reason for this absence is not obvious, but may be related to the fact that direct receptor-mediated stimulation of adenylate cyclase is also difficult to detect in membrane preparations. Alternatively, a requirement for some soluble factor may preclude the detection of any augmentation in isolated plasma membranes.

Since the augmentation response is a functional measure of receptor activity, this system can be used to screen for potential α -adrenergic and GABA_B receptor agonists and antagonists, which cannot be identified with receptor binding analysis. Brain slice preparations can also disclose the biochemical consequences of agents like phorbol esters that act at intracellular sites. And while the physiological relevance of receptor-mediated cAMP augmentation is still unknown, brain slice studies have hinted that the pharmacological manipulation of neuromodulator receptors might hold considerable therapeutic potential. □

William Karbon is at the Department of Pharmacology, Yale University School of Medicine, PO Box 3333, New Haven, Connecticut 06510, USA. Salvatore Enna is research director at Nova Pharmaceutical Corp, 5210 Eastern Avenue, Baltimore, Maryland 21224, USA.

- Ross, E.M. & Gilman, A.G. *Rev. Biochem.* **49**, 553–564 (1980).
- Berridge, M.J. *Biochem. J.* **220**, 2625–2628 (1984).
- Duman, R.S. *et al. J. Neurochem.* **47**, 800–810 (1986).
- Perkins, J.P. & Moore, M.M. *J. Pharmac. exp. Ther.* **185**, 371–378 (1973).
- Pilc, A. & Enna, S.J. *J. Pharmac. exp. Ther.* **237**, 725–730 (1986).
- Enna, S.J. & Karbon, E.W. in *Benzodiazepine/GABA Receptors and Chloride Channels* (eds Olsen, R.W. & Venter, J.C.) 41–56 (Liss, New York, 1986).
- Karbon, E.W. & Enna, S.J. *Molec. Pharmacol.* **27**, 53–59 (1985).
- Schwabe, U. & Daly, J.W. *J. Pharmac. exp. Ther.* **202**, 134–143 (1977).
- Karbon, E.W. *et al. J. Neurochem.* (in the press).
- Castagna, M. *et al. J. biol. Chem.* **257**, 7847–7851 (1982).
- Enna, S.J. & Karbon, E.W. *Trends pharmac. Sci.* (in the press).

The news in neuroscience

November's meeting of the American Society for Neuroscience will bring a host of novel products to Washington.

A **brain slice chamber** with interchangeable top units allows testing for drug action by alternative techniques (Reader Service No. 100). Medical Systems Corp makes two tops to accompany the single base unit: a Haas design that uses stable semi-submersion techniques, and a Zbiciz top that maintains tissue slices submerged under a constant flow of perfusing liquid. The company claims its instrument can achieve cell longevity of 10 hours or more. For either top unit, Medical Systems has a temperature controller that regulates between 30 and $40 \pm 0.2^\circ\text{C}$.

ADVERTISEMENTS

LIPID PRODUCTS

top quality lipids for biomedical research
17 years experience supplying lipids to top research laboratories throughout the world

For free catalogue contact:
Lipid Products, Nutfield Nurseries
South Nutfield, Redhill
Surrey RH1 5PG, UK
Tel: (073 782) 3277

Reader Service No.37

Versatile Tissue Culture Incubator

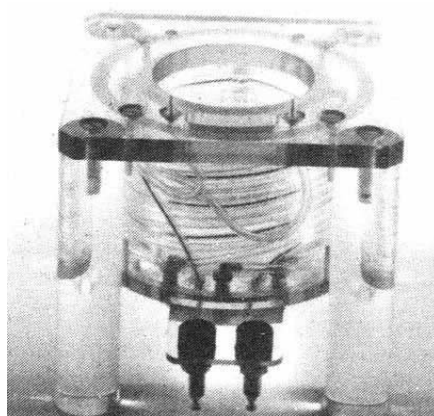
Modular Incubator Chamber (the Cell/Sphere™) for all tissue cultures, aerobic or anaerobic.

Low Cost



Eliminates evaporation loss and contamination. Easy to use – flush with gas mixture, seal, place at appropriate temperature. Stackable, see-through. Quantity discounts. billups-rothenberg, inc., pob 977, Del Mar, CA USA 92014. Call 619-755-3309 or contact your Flow Laboratories representative.

Reader Service No.7

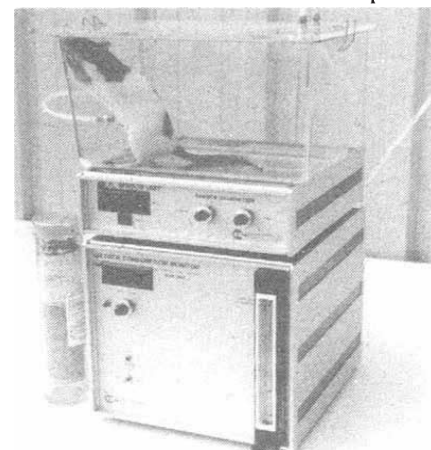


Takes the headaches out of brain slicing.

For **rapid kinetics studies**, Intracel Ltd supplies Bio-Logic instrumentation and software from France in three different configurations (Reader Service No. 101). The stopped flow, rapid filtration and quench flow systems are all available with microprocessor control, and are priced between £9,600 and £18,000 (UK). Intracel says programmable stepping motors and the unusual design of the mixing chambers reduce dead volume and dead time.

Three separate background measurements and all forms of evoked potential averaging are part of an **EEG analysis package** from Biodata (Reader Service No. 102). The computer-aided CEAN 400 system, which includes the hardware, software and peripherals for various applications, simplifies the analysis of extended EEG records typical of drug and sleep research, according to the company. Priced at approximately \$25,000 (US), Biodata's instrument can be integrated with existing EEG equipment, or sold with its own amplifiers and stimulators.

Any animal or plant that will fit is fair game for Columbus Instruments' economy **oxygen consumption monitor** (Reader Service No. 103). The company's \$7,926 (US) system, with dimensions of $11 \times 15 \times 13$ inches, is sensitive to percentage of oxygen, not oxygen partial pressure, so standardized values of consumption are more accessible. Although this model requires operator assistance, it can be automated with connection to a computer.



Measuring O₂ consumption by open circuit calorimetry.

Columbus also has a fully computerized IBM PC/XT version of its monitor.

Eight more **antibodies for neurological investigations** are now available from ICN Biomedicals (Reader Service No. 104). The company says its line addresses studies of neural tissue and cell differentiation, typing of neural tumours and research on disorders and diseases such as Alzheimers disease. ICN draws attention to its Alzheimers agents in particular, including antisera to the paired helical filaments (PHF) and to microtubule-associated proteins. □

These notes are compiled by Karen Wright from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

Fluoro Plastic Devices for Trace Analysis



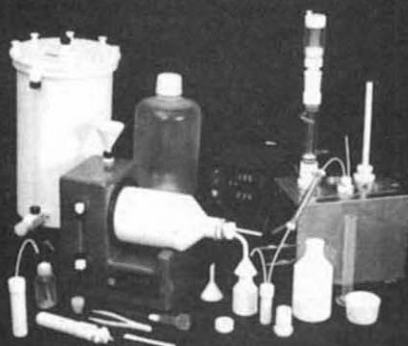
Teflon® PTFE, PFA and FEP

excellent materials for non-contaminational working with pure and aggressive substances at high temperature

- „Subboiling“ Acid Purifying also for hydro fluoric acid
- Storage vessels for liquids (5 to 45 litres)
- Decomposition Systems under pressure, reflux conditions
- Separation of volatile compounds up to 260°C
- Sample preparation (phase separators, gas sample valve)

Berghof GmbH · Abt. Labortechnik

D-7412 Eningen · Harretstr. 1 · Tel. 07121/89010 · Telex 729529



Reader Service No.25